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Dissecting the functional role of gonadotropin releasing hormone neurons in sex-specific olfactory processing

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ENGLISH ABSTRACT

Pheromones influence reproductive physiology and behaviors in various species, humans included. Indeed, pheromone exposure in rodents accelerates sexual maturation, promotes estrous synchronization in females, and induces a fast increase of serum LH levels in both sexes. The role of gonadotropin-releasing hormone (GnRH) neurons as the final neural output controlling reproduction fitness and fertility is well established among the different vertebrates. GnRH neurons are also known to integrate sex-related olfactory information to coordinate and optimize reproduction through another network involving olfactory and limbic areas in the brain. In addition, an extra-hypothalamic GnRH neuronal population has been identified, in both rodents and humans, in areas dedicated to olfactory processing and more specifically in the olfactory bulbs (OB). We named this population OB GnRH neurons. We hypothesized that this newly identified population of GnRH neurons may convey olfactory and/or pheromonal information to participate in the neuroendocrine and behavioral responses controlling reproduction. Using viral cell tracing and whole-head tissue-clearing and 3D-imaging, we show that OB GnRH neurons extend long projections into the vomeronasal organ, the main olfactory epithelium, and the median eminence. We confirmed by single-cell RNA sequencing of the entire olfactory bulb population that OB GnRH neurons express olfactory and pheromone receptors suggesting a possible detection of olfactory cues by the OB GnRH neurons. We then investigated whether OB GnRH neurons can be activated by opposite-sex smell. Using *in vivo* calcium imaging coupled with two-photon microscopy we confirmed that olfactory and pheromonal stimulation can activate the OB GnRH somata and their processes. To confirm that this activation participates in the neuroendocrine responses, we tested whether OB GnRH neurons promote LH release. Our results showed that the chemogenetic activation of OB GnRH neurons triggers LH release in male mice. Using a combination of virogenetic experiments and electrophysiological recordings on hypothalamic slices we demonstrate that OB GnRH neurons are also connected to the GnRH population located in the rostral preoptic area and that chemogenetic activation of OB GnRH neurons promote an increase of POA GnRH firing activity. Finally, the function of OB GnRH neurons was investigated through multiple olfactory and social behavior tests using chemogenetic and neuronal ablation approaches. Remarkably, our data demonstrate that male preference for female odors is enhanced upon chemogenetic activation of OB GnRH neurons, and,

oppositely, it is impaired after genetic inhibition or ablation of these cells. Our data also suggest that these behavioral responses rely on GnRH signaling in the posterodorsal medial amygdala and the preoptic area. Overall, our study highlights a novel role for olfactory GnRH neurons as a central regulatory hub linking pheromonal stimulation with reproductive functions.

FRENCH ABSTRACT

Les phéromones influencent la physiologie et les comportements reproductifs chez diverses espèces, y compris l'homme. En effet, chez les rongeurs l'exposition aux phéromones accélère la maturation sexuelle, favorise la synchronisation de l'œstrus chez les femelles et induit une augmentation rapide des niveaux de LH sérique chez les deux sexes. Le rôle des neurones à gonadolibérine (GnRH) contrôlant l'aptitude à la reproduction et la fertilité, est bien établi chez les différents vertébrés. Les neurones à GnRH sont également connus pour intégrer les informations olfactives liées au sexe afin de coordonner et d'optimiser la reproduction par le biais d'un autre réseau impliquant les zones olfactives et limbiques du cerveau. Par ailleurs, une population de neurones à GnRH extra-hypothalamique a été identifiée, à la fois chez les rongeurs et chez l'homme, au sein des bulbes olfactifs, dans des zones dédiées au traitement des informations olfactives. Nous avons nommé cette population les neurones à GnRH olfactifs. Nous avons émis l'hypothèse que cette population de neurones à GnRH nouvellement identifiée pourrait transmettre des informations olfactives et/ou phéromonales afin de participer aux réponses neuroendocrines contrôlant la reproduction. En utilisant des traceurs viraux, la transparisation de la tête entière et l'imagerie 3D, nous avons montré que les neurones à GnRH olfactifs possèdent de longues projections atteignant l'organe voméronasal (VNO), l'épithélium olfactif et l'éminence médiane. Nous avons confirmé par séquençage ARN à noyau unique réalisé sur l'ensemble de la population du bulbe olfactif que les neurones à GnRH olfactifs expriment des récepteurs olfactifs et des récepteurs de phéromones, ce qui suggère une possible détection des signaux olfactifs par les neurones à GnRH olfactifs. Nous avons ensuite cherché à savoir si les neurones à GnRH olfactifs pouvaient être activés par des odeurs de sexe opposé. En utilisant l'imagerie calcique *in vivo* couplée à la microscopie bi-photonique, nous avons confirmé que les stimulations olfactives et phéromonales issues du sexe opposé peuvent activer les corps cellulaires et les prolongements des neurones à GnRH olfactifs. Afin de confirmer que cette activation participe aux réponses neuroendocrines, nous avons testé si les neurones à GnRH olfactifs favorisent la libération de LH. Nos résultats montrent que l'activation chimiogénétique des neurones à GnRH olfactifs déclenche la libération de LH chez les souris mâles et cette libération de LH est abolie lors de l'inhibition de ces neurones. En utilisant un virus DREADD activateur et des enregistrements électrophysiologiques sur des tranches de cerveau, nous avons démontré

que les neurones à GnRH olfactifs sont connectés à la population de neurones à GnRH située dans l'aire pré-optique hypothalamique car l'activation des neurones à GnRH olfactifs déclenche une augmentation de l'activité des neurones à GnRH hypothalamiques. Enfin, la fonction des neurones à GnRH olfactifs a été étudiée par le biais de multiples tests olfactifs et de comportements sociaux en utilisant des approches chimiogénétiques et d'ablation neuronale. De façon remarquable, nos données démontrent que les neurones à GnRH olfactifs sont essentiels au décodage des informations olfactives sociales et plus particulièrement jouent un rôle dans la préférence olfactive. En effet, la préférence des mâles pour les odeurs en provenance du sexe opposé est renforcée par l'activation chimiogénétique des neurones à GnRH olfactifs, et altérée par l'inhibition génétique ou l'ablation de ces cellules. Nos résultats suggèrent également que les neurones à GnRH olfactifs facilitent les comportements de reproduction en acheminant des informations olfactives au sein de la partie postéro-dorsale de l'amygdale médiale et de l'aire pré-optique. Dans l'ensemble, notre étude met en évidence un rôle nouveau pour les neurones à GnRH olfactifs en tant que régulateurs centraux reliant la stimulation olfactive aux fonctions reproductives.

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ABBREVIATIONS

ACN: anterior cortical nucleus

AHA: anterior hypothalamic area

AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AOB: accessory olfactory bulb

AON: accessory olfactory nucleus

AOS: accessory olfactory system

AR: androgen receptor

ARC: arcuate nucleus

AVPV: anteroventral periventricular nucleus

BNST: bed nucleus of the stria terminalis

pBNST: posterior part of the bed nucleus of the stria terminalis

CX: cortex

DREADD: designer receptors exclusively activated by designer drugs

dSA: deep Short Axon Cells

EC: entorhinal cortex

EPL: external plexiform layer

ERα: estrogen receptor α

ERβ: estrogen receptor β

FACS: fluorescent activated cell sorting

FSH: follicle-stimulating-hormone

GABA: γ-aminobutyric acid

GAP: gonadotropin-releasing hormone associated peptide

GCL: granular cell layer

GFP: green-fluorescent protein

GL: glomerular cell layer

GnRH: gonadotropin-releasing hormone

GnRH-R: gonadotropin-releasing hormone receptor

GO: Gene Ontology

GPCR: G-protein coupled receptor

GrL: granular cell layer

GT: ganglion terminal

HPG: hypothalamic-pituitary-gonadal

IHH: idiopathic hypogonadotropic hypogonadism

IN: inhibitory neurons

Kiss1: kisspeptin

Kiss1R: kisspeptin receptor

KOR: kappa opioid receptor

LA: lateral amygdala

LC: locus coeruleus

LH: luteinizing hormone

LatH: lateral hypothalamus

LS: lateral septum

MBH: medio-basal hypothalamus

MCL: mitral cell layer

ME: median eminence

MeA: medial amygdala

MeAa: anterior medial amygdala

MeAp: posterior medial amygdala

MePO: median preoptic nucleus

MOB: main olfactory bulb

MOE: main olfactory epithelium

MOS: main olfactory system

MPOA: medial preoptic area

MS: medial septum

M/T: mitral/tufted cells

MTMT: (methylthio)methanethiol

MUPs: major urinary proteins

Nac: nucleus accumbens

NKB: neurokinin B

NLOT: nucleus of the lateral olfactory tract

NMDA: N-methyl-D-aspartate

nNOS: neuronal nitric oxide synthase

NO: nitric-oxide

OB: olfactory bulb

OE: olfactory epithelium

OEC: olfactory ensheathing cells

ONL: olfactory nerve layer

ORs: olfactory receptors

OSN: olfactory sensory neurons

OT: olfactory tubercle

OVLT: organum vasculosum of the lamina terminalis

PAG: periaqueductal grey

PC: piriform cortex

PFC: prefrontal cortex

PG: periglomerular cells

PGE_2: prostaglandin E2

PLCN: posterolateral cortical amygdaloid nucleus

PMCo: posteromedial cortical amygdaloid nucleus

PMCN: posterior-medial cortical epithelium

POA: preoptic area

PR: progesterone receptor

rPOA: rostral preotic area

RP3V: rostral periventricular area of the third ventricle

sSA: superficial Short Axon Cells

SVZ: subventricular zone

TAARs: trace amine-associated receptors

TMT: 2,5-dihydro-2,4,5-trimethylthiazoline

TT: tenia tecta

UMAP : Uniform Manifold Approximation and Projection

vGLUT2: vesicular glutamate transporter type 2

VMH: ventromedial hypothalamus

VNO: vomeronasal organ

VTA: ventral tegmental area

3V: third ventricle

Chapter I: Reproduction and the Gonadotrophin-releasing hormone neurons

I. The hypothalamic network governing reproduction

Reproduction allows the perpetuation of all living beings and its cellular control by different components of the organism guarantees the survival of the species. The mechanisms underlying reproduction are highly conserved in vertebrates and rely on the proper function of the hypothalamic-pituitary-gonadal (HPG) axis. As its name indicates, it depends on the cross-talk between three main elements: the hypothalamus, the pituitary, and the gonads (**Figure 1**). The hypothalamus, a small region found at the base of the mammalian brain, is involved in the fine control of life-dependent functions and body homeostasis. To this end, it exerts diverse functions such as the regulation of energy balance, thermoregulation, stress responses, social behaviors and reproduction. The hypothalamus is composed of several nuclei and areas, hosting neurons and circuits, which are conserved throughout evolution due to their importance in basic life-functions (Burbridge et al., 2016). The hypothalamus is host to Gonadotropin-Releasing Hormone (GnRH) neurons, onto which converge several internal and external cues relevant to the control of the reproductive function. GnRH neurons integrate these cues to elicit an appropriate response (Herbison, 2006) and finely regulate the production of GnRH, and its secretion, at the level of the median eminence (ME) (Wray and Hoffman, 1986).

The first evidences of a neuroendocrine control of reproduction emerged at the beginning of the 20th century. Indeed, in the 1920s, numerous studies showed that hypothalamic lesions lead to genital atrophy and result in sexual impairment. It has been demonstrated that damaging the hypothalamus, the pituitary stalk or the median eminence lead to genital atrophy and ovarian disturbances such as a loss of cyclicity. In addition, ablation and replacement studies highlighted the role of the pituitary in gonadal growth and estrous cyclicity (Harris, 1948). These evidences support the hypothesis of a substance transmitted from the hypothalamus through the hypophysial portal vessels to the pituitary gland. In 1930, Popa demonstrated the existence of a portal system between the pituitary and the hypothalamic region (Popa, 1930). Then, in 1937, two papers written by Francis Marshall, Ernest Verney and Geoffrey Harris provided evidences of the role of the central nervous system, including the pituitary gland, in the control of reproduction and fertility. Indeed, brain stimulations, and stimulation of the pituitary gland, induced ovulation in female rabbits

(Harris, 1937). Although the pathways by which the pituitary gland was stimulated remained elusive, Harris hypothesized in 1948 that “a nervous stimulus may cause the release of a substance into the capillary sinusoids of the median eminence, this substance then being transported via the hypophysial portal vessels to excite or inhibit the pars distalis" (Harris, 1948). This quest was furthered in 1971 by Andrew Schally and Roger Guillemin, who published the primary structure of a decapeptide called luteinizing hormone-releasing hormone (LHRH), capable of promoting the secretion of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH). As this decapeptide was able to stimulate the secretion of both gonadotropins, it was later renamed gonadotropin-releasing hormone or GnRH. Julien Barry, in Lille, was the first to produce an antibody directed against GnRH usable for immunohistochemistry, which allowed to highlight the presence of GnRH cell bodies and fibers along the hypothalamus and the median eminence in a wide range of vertebrates including humans (Barry et al., 1973).

The median eminence is located on the ventral surface of the tuberal region of the hypothalamus and continues into the pituitary gland. The ME is a circumventricular organ, characterized by fenestrated capillaries that facilitate the diffusion of circulating molecules into the brain parenchyma. In the ME, as it was previously mentioned, GnRH neurons terminals reach the capillaries of the hypophyseal-portal circulation and release GnRH to stimulate the pituitary gland.

The pituitary gland, located beneath the hypothalamus and linked to it by the hypophyseal portal circulation, is a relatively small structure composed of an anterior and a posterior part termed adenohypophysis and neurohypophysis, correspondingly. The neurohypophysis consists primarily of oxytocin and vasopressin neuronal axons originated from the paraventricular and supraoptic nuclei, while the adenohypophysis encompasses various endocrine cells, such as thyrotropes, somatotrophs, lactotrophs, corticotropes, and gonadotrophs, which the latter represent 10 to 15% percent of the endocrine glandular population (Stamatiades and Kaiser, 2018). GnRH peptides released into the portal circulation target the gonadotroph cells of the anterior pituitary. These cells highly express the GnRH receptor (GnRH-R), which is a specific G-protein coupled receptor that coordinates the secretory machinery of these cells (Thompson and Kaiser, 2014). GnRH is released in episodic

pulses, that are critical for the proper functioning of the HPG axis (Belchetz et al., 1978). Variations in GnRH pulsatile secretion coordinate the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the gonadotrophs. Low frequencies of GnRH pulses stimulate FSH production, while high frequencies preferentially stimulate LH production (Wildt et al., 1981; Stamatiades and Kaiser, 2018). These gonadotropins enter the general circulation and act downstream on the gonads (ovaries and testes) to regulate gonadal maturation, folliculogenesis, ovulation, spermatogenesis, and steroidogenesis (Burger et al., 2004).

LH and FSH serve as regulators of ovarian and testicular function by binding to specific gonadotrophic receptors of the rhodopsin/2 adrenergic-R family of G protein-coupled receptors (GPCRs) located on the gonads' membrane (Rabinovici, 1993). LH in the testis promotes testosterone production by Leydig cells, leading to sexual anabolic actions and spermatogenesis maintenance. Meanwhile, FSH binds to its receptor on Sertoli cells to induce differentiation, growth, and spermatogenesis. In males, gonadotrophins are necessary for sustaining steroidogenesis and spermatogenesis (Kaprara and Huhtaniemi, 2018). LH and FSH actions in the ovaries depend on the developmental stage of the follicles, with only the terminal stage of follicle development being reliant on gonadotropin actions. FSH enables the recruitment of pre-ovulatory follicles and facilitates follicular growth. However, a follicle becomes dominant upon LH action. Finally, both gonadotrophins promote the production of steroid hormones (estradiol, progesterone and testosterone) (Mcgee and Hsueh, 2000).

Finally, steroid hormones regulate the function of the HPG axis by inducing sex-dependent feedbacks at the central level.

1.1 Negative feedback from sex steroids in both sexes

A negative feedback mechanism connects the different components of the HPG axis. Gonadal sex steroid hormones (testosterone, estradiol, progesterone) are secreted into the general circulation, where they exert their actions not only on the gonads but also on the hypothalamus and the pituitary gland by mostly down-regulating GnRH secretion (Herbison,

2016). GnRH neurons lack the necessary steroid hormone receptors to impinge feedback information. Instead, other neuronal populations located in the hypothalamus, upstream to GnRH neurons, and the pituitary gland regulate GnRH neuron activity and secretory function throughout the reproductive lifespan through distinct mechanisms describe further in this chapter.

1.2 Positive feedback from sex steroids in females

The ovarian cycle refers to changes occurring in the ovaries leading to the maturation of the follicles to trigger ovulation and the degradation of the mature follicle(s), named corpus luteum, in absence of fertilization. The ovarian cycle is divided in three phases: the follicular phase, ovulation and the luteal phase. The ovarian cycle starts with a gonadotropin-independent recruitment toward growth into pre-antral follicles. Pre-antral follicles highly express FSH receptors in the granulosa cell layer, which in turn secretes increasing amounts of estrogen into the general circulation. The follicle that advances further in the developing stages becomes the dominant follicle, which secretes more estrogens and highly expresses LH receptors.

Mounting estrogens levels reach the brain and the GnRH neuronal network leading to a switch from negative feedback to positive feedback that enhances GnRH secretion. As GnRH and LH have a one-to-one ration relation (Belchetz et al., 1978), this surge of GnRH readily triggers an LH surge, which acts downstream in the ovaries. Then, the dominant pre-ovulatory follicle, which highly expresses LH receptors, responds to the LH surge by triggering ovulation, the luteinization process and greater progesterone synthesis.

This positive feedback mechanism is essential for reproduction as proper ovulatory rates contribute to greater female reproductive fitness (Herbison, 2016).

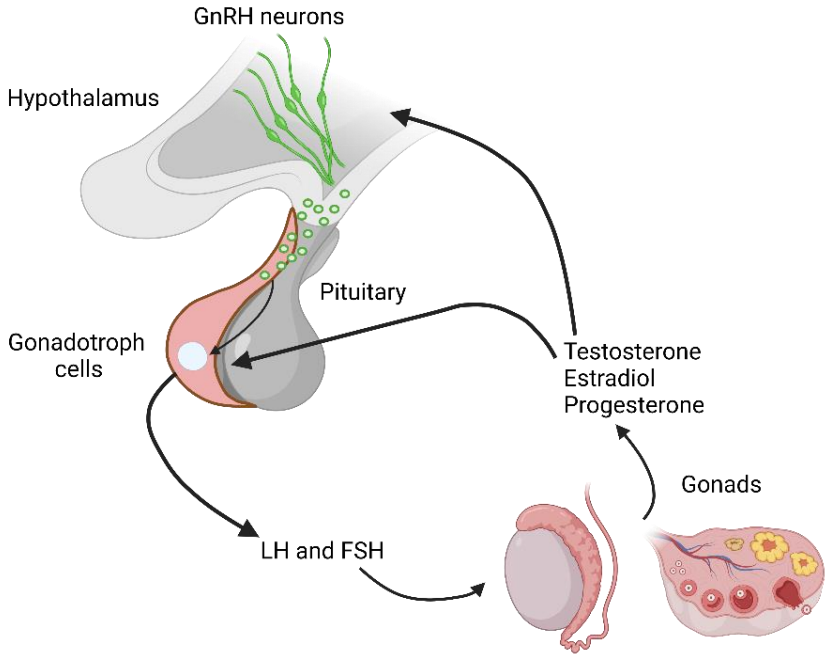

Figure 1: Hypothalamic-Pituitary-Gonadal axis. Neuroendocrine GnRH neurons project to the median eminence and secrete episodic pulses of GnRH into the hypophyseal portal system. GnRH signaling in the pituitary gland coordinates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from gonadotroph cells. LH and FSH are critical for gonadal maturation and sex steroid hormone production. In turn, sex steroids promote secondary sex characteristics in peripheral tissues and regulate GnRH neuron functioning via neuroendocrine feedback mechanisms.

II. GnRH biosynthesis

2.1 GnRH from gene to peptide

2.1.1 GnRH-I gene

The GnRH hormone is a decapeptide synthesized by specialized neurons. The gene coding for GnRH is located on chromosome 8 in humans and chromosome 14 in mice. The gene is composed of four exons and three introns. The GnRH decapeptide is derived from exon two. In addition, exons two, three and four, produce another peptide named the GnRH-associated peptide (GAP) (Seeburg and Adelman, 1984). The transcription of the GnRH gene generates a polypeptide precursor that is cleaved to yield a mature transcript. GnRH neurons are known

to contain numerous *Gnrh* mRNAs at levels comparable to housekeeping genes. The transcription of the GnRH gene is rapid, taking about 18 minutes and depends on internal and external factors, such as sex-steroids positive feedback leading to a 40% rise in GnRH transcription (Yeo et al., 1996; Herbison, 2006).

2.1.2 GnRH-I peptide

The mature GnRH sequence is derived from a preprohormone composed of 92 amino acids. The prohormone sequence comprises 23 amino-acids that are cleaved from the N-terminal of the preprohormone sequence. Within the prohormone, both sequences of the GnRH-I and the GnRH-I-associated peptide are present. The separation of the GAP from the GnRH-I sequence occurs through the cleavage of a specific sequence composed of three amino acids. The conversion of glutamate to pyroglutamate and the amidation of the glycine at the N-terminal and C-terminal, respectively, of the GnRH decapeptide are essential for obtaining the mature GnRH sequence. The active form of GnRH has a horse-shoe shape. Finally, the decapeptide is packaged in storage granules and transported down to the axon, where it is later secreted in the portal circulation (Millar et al., 1989).

2.2 Other GnRH molecules

Different GnRH forms have been identified in vertebrates, however in mammals only three forms are described, the GnRH-I, GnRH-II and the GnRH-III (Millar, 2005).

The GnRH-I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) commonly named "GnRH", is the hypophysiotropic form, conserved throughout evolution in vertebrates. The guinea pig is unique among mammals in its utilization of a GnRH decapeptide that differs by a single amino-acid from the classical GnRH-I decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-**Val**-Arg-Pro-Gly-NH2) (Schally et al., 1971; Matsuo et al.,1971; Baba et al., 1971).

GnRH-II, the second form of GnRH, was initially isolated from chicken hypothalamic extract and remains the most conserved form of this hormone, with a decapeptide sequence differing from GnRH-I by only three amino acids (pGlu-His-Trp-Ser-**His**-Gly-**Trp**-**Tyr**-Pro-Gly-NH_2) (Miyamoto et al., 1984). The release of GnRH-II remains unobserved in GnRH-I neurons, implying the production of this hormone by another population of GnRH. The function of GnRH-II in regulating the neuroendocrine aspects of reproduction remains poorly understood.

The wide distribution of GnRH-II throughout the peripheral and central nervous systems suggests a neurotransmitter role. Although the investigation of GnRH-II within the portal circulation was unsuccessful, its role in promoting gonadotropin release remains a topic of exploration (Herbison, 2006). Additionally, GnRH-II functions as a weak agonist of the GnRH-I receptor, as its binding sites differ from those of Type I GnRH receptors but exhibit a high affinity for Type II GnRH receptors (Millar et al., 1987). Moreover, it should be noted that this receptor has not been identified in humans, chimpanzees, horses, sheep, rats, or mice, but has been found in marmosets, rhesus monkeys, and pigs (Morgan et al., 2003). GnRH II has no apparent effect on gonadotropin release, but evidence suggests it plays a role in food intake (Herbison, 2006). Finally, it is possible that GnRH-II is involved in sexual arousal due to its location in areas relevant to reproductive behavior (Rissman et al., 1997; Millar, 2003).

GnRH-III, the active GnRH form in fish, was identified in lamprey and can be found in various vertebrate species (pGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH2) (White et al., 1994). Although GnRH-III is present in the human hypothalamus, it has been revealed to be a weak agonist of the GnRH-I receptor, questioning its role in gonadotropin release (Herbison, 2006). The precise role of GnRH-III is yet to be determined.

To conclude, in mammals, the GnRH-I is the relevant form exerting a major neuroendocrine control of reproduction. (Millar, 2005).

III. The GnRH neurons

3.1 The peculiar origin of GnRH neurons

The GnRH neuronal population comprises about 800-1000 neurons in the entire brain in adult rodents and about 2,000 neurons in human hypothalamus (Casoni et al., 2016; Skrapits and Hrabovszky, 2018). The ontogenesis of GnRH neurons is unique, unlike most neurons, GnRH neurons originate outside the central nervous system within the olfactory placode and then, during embryonic development, migrate to the brain (Schwanzel-fukuda, 1989; Wray et al., 1989). At embryonic day 11 (E11), GnRH immunoreactivity is initially observed in the nasal epithelium and in the medial part of the olfactory placode, which ultimately develops into the future vomeronasal organ (VNO) in fetal rhesus monkeys and mice (Schwanzel-Fukuda and

Pfaff, 1990a). During this developmental stage, most GnRH neurons begin to differentiate from their stem cell precursors, showing an oval shape oriented perpendicularly to the luminal section of the epithelium. At times, a single GnRH-immunoreactive process extends to the VNO lumen (Schwanzel-fukuda, 1989). Between E12 and E13, a large amount of GnRH neurons is located in the placode's epithelia. These GnRH neurons are associated with terminal and vomeronasal nerves on the nasal septum that head towards the forebrain, passing through the cribriform plate. By E14, the presence of GnRH neurons is observed in the preoptic area and hypothalamus. Finally, by E16, the majority of GnRH neurons are located in the preoptic and hypothalamic areas (Schwanzel-fukuda, 1989)(**Figure 2**).

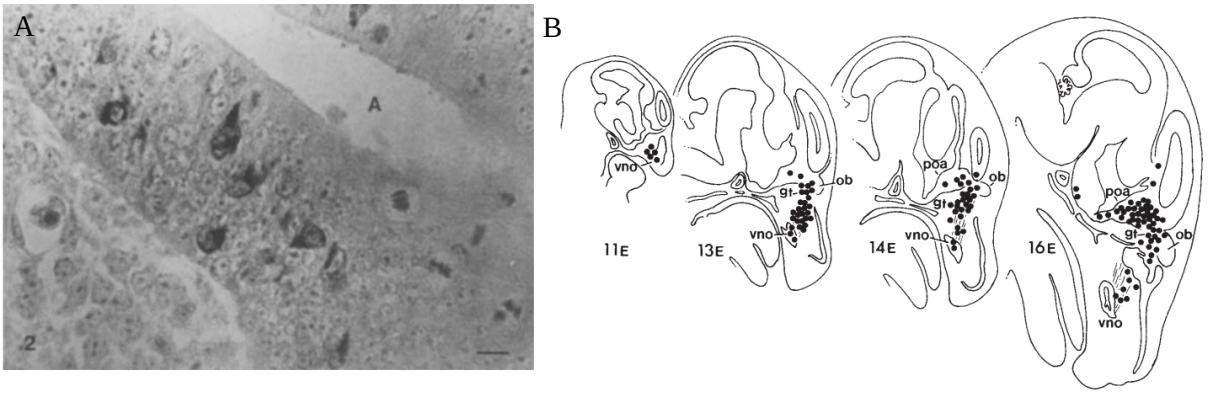

Figure 2: The migratory road of GnRH neurons from the olfactory placode to the forebrain in mouse. **(A)** 8µm sagittal section of the medial olfactory placode at embryonic day 11th. GnRH immuno-reactive neurons originate perpendicular to the vomeronasal lumen. A is the lumen of the VNO. **(B)** Drawing in sagittal plane of whole head of fetal mice at embryonic days 11, 13, 14 and 16. The black dots represent GnRH neurons. GnRH neurons originate from the medial olfactory placode at E11. At E13, the majority of GnRH neurons are in close apposition to the terminal and vomeronasal nerves on the nasal septum, migrating from the olfactory placode to the forebrain. GnRH neurons reach the preoptic area at E14. Most GnRH neurons are located in the preoptic and hypothalamic areas at E16. Scale bar = 10µm. vno: vomeronasal organ; gt: ganglion terminal; ob: olfactory bulb; poa: preoptic area. Adapted from Schwanzel-fukuda, 1989 and Schwanzel-Fukuda and Pfaff, 1990.

Interestingly, the study revealed a decrease in the number of GnRH neurons in the olfactory placode and an increase in the brain, lending support to the hypothesis of an active migration process from the olfactory placode to the hypothalamus. Further research indicates that GnRH neurons initiate migration to the developing forebrain at embryonic day E11.5 in mice and around the fifth week of fetal development in humans (Casoni et al., 2016). Additionally, GnRH neurons migration is generally completed by 16 days of gestation in mice (Schwanzel-Fukuda and Pfaff, 1990).

3.2 Molecular mechanisms regulating GnRH migration

Proper GnRH development and signaling are essential to achieve sexual maturity and maintain reproductive capacity. Disruptions in this neuronal system result in sexual immaturity and infertility (Wray and Hoffman, 1986) and are associated with a human genetic disease, known as idiopathic hypogonadotropic hypogonadism (IHH). IHH is marked by GnRH deficiency, lack of gonadal maturation, puberty onset delays or absence, and complete infertility (Boehm et al., 2015). When IHH is accompanied by anosmia, in 60% of the cases, it is classified as Kallmann syndrome. IHH are rare disorders, with about 60% of sporadic cases and 40% of familial cases (Hu and Bouloux, 2011).

As mentioned before, GnRH neurons originate in the olfactory placode, migrate along vomeronasal and terminal nerves, along the nasal septum, cross the cribriform plate, pass through the olfactory bulbs to reach the preoptic area and the hypothalamus. Four critical steps are needed for proper migration of GnRH neurons: 1) adhesion of GnRH neurons to axons; 2) guidance of GnRH neurons to the forebrain; 3) extension of GnRH processes in the hypothalamus; 4) cessation of GnRH hypothalamic migration and setting (Wierman et al., 2011). The completion of each of these steps requires multiple factors including adhesion molecules, guidance cues, growth and transcription factors to regulate the correct fate and implementation of GnRH neurons (**Figure 3**).

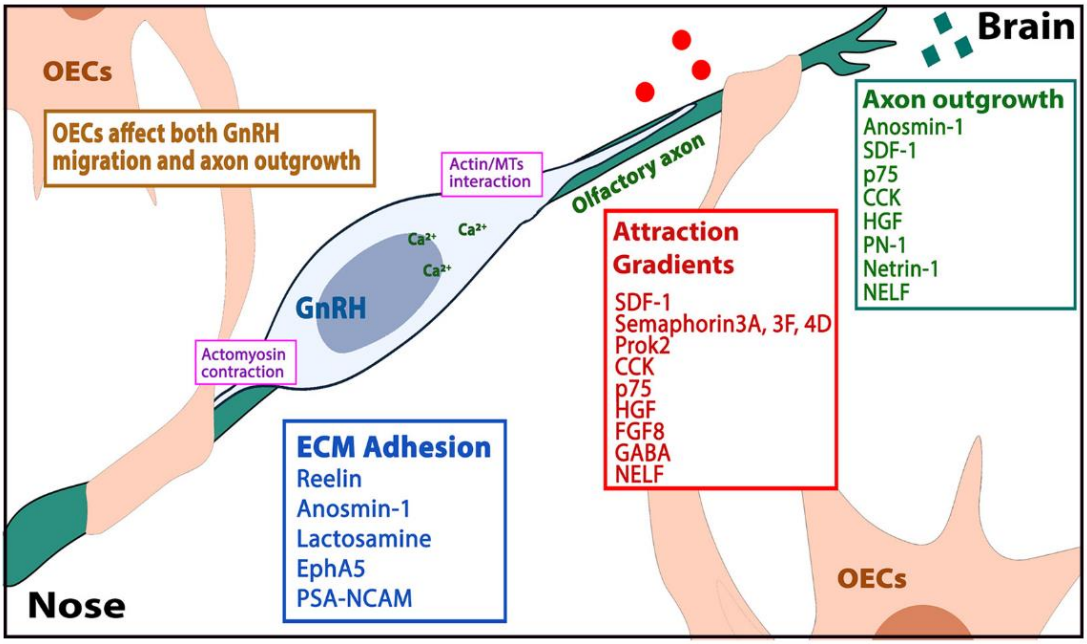

Figure 3: **Molecules regulating GnRH neuronal migration**.

Schematic representing a GnRH neuron along an olfactory axon, enwrapped by OEC. Molecules involved in ECM adhesion, attraction gradients, axon outgrowth and migration are shown in different boxes. Adapted from (Cho et al., 2019).

3.3 Distribution of GnRH neurons

The unique development of GnRH neurons in mammals results in a scattered continuum of GnRH cell bodies along the migratory stream in the forebrain. The GnRH distribution refers to an inverted Y pattern with the midline extending from the olfactory bulbs to the medial septum (MS) which separates at the beginning of the third ventricle within the rostral preoptic area (rPOA). From there, two arms extend through the anterior hypothalamic area (AHA) and the medio basal hypothalamus (MBH) (Herbison, 2015)(**Figure 4**). In mice, rats and sheep, almost all GnRH neurons are scattered along this inverted Y pattern. In guinea pigs, monkeys and humans the GnRH distribution differs with more GnRH neurons located caudally (Herbison, 2015). Unlike in mice, where the number of GnRH neurons decreases between fetal development and adulthood, the complete GnRH population of humans survives until adulthood, with minimal loss (Casoni et al., 2016; Schwanzel-Fukuda and Pfaff, 1990b; Wray et al., 1989). During fetal development in humans, whole brain analysis and 3D imaging has

revealed that an average of 10,000 GnRH neurons throughout the entire brain with no distinction between sexes (Casoni et al., 2016). By the end of the third trimester, GnRH cell bodies complete their migration with approximately 2,000 neurons located in the hypothalamus and 8,000 neurons dispersed in various extra-hypothalamic regions (Casoni et al., 2016). More recent data, unexpectedly estimate high number of extrahypothalamic GnRH cell bodies (150,000 to 200,000) distributed in widespread brain areas (Skrapits et al., 2021). Extrahypothalamic GnRH populations can be found in several regions, including the bed nucleus of the stria terminalis (BNST), the subfornical organ, the corpus callosum, the cortex, and the olfactory bulbs (Casoni et al., 2016; Herbison, 2015; Skrapits et al., 2021). In the olfactory areas, and more particularly in the olfactory bulbs, a large number of GnRH immuno-reactive cell bodies and fibers have been described (Jennes, 1986). A ring-like GnRH structure composed of GnRH cell bodies and fibers has been identified encircling the olfactory bulbs in adult mice as well as in human fetuses (Casoni et al., 2016). However, the putative function of these neurons remains to be assessed.

3.4 GnRH neuron morphology

Different articles show that GnRH neurons exhibit a stereotypic morphology across the brain with a cell body, a single axon, and a single dendrite. Interestingly, GnRH dendrites are longer than those of classical neurons, measuring over 2,000 µm (Herbison, 2015). The GnRH neuronal morphology observed in adult rodents derives from anatomical consolidation during postnatal development. Indeed, in infants, GnRH somatic area, total dendritic length and total number of dendrite branches are higher than in adult rodents, suggesting a spatial selective remodeling of the neurons across development (Ybarra et al., 2011). In adults, most GnRH neurons exhibit a bipolar morphology, with a single dendrite and a single axon arising from the soma. The dendrite is oriented with a specific orientation, at 180° from the axon. Conversely, in prepubertal rodents, there are one to seven primary dendrites (with an average of 2.2) arising from the soma with variable angles (Ybarra et al., 2011). This GnRH neuronal remodeling could be part of the control of the onset of puberty in rodents. However, it should be noted that some GnRH neurons are multipolar in adult sheep and humans, as reported by Skrapits et al. (2021).

In addition, GnRH neuronal orientation is dependent on the location of the neuron in the brain. In the medial septum, GnRH neurons possess a vertical orientation, whereas closer to the medio-basal hypothalamus, they exhibit a horizontal orientation. Within the medial septum GnRH fibers are running parallels and are in close appositions in the rPOA and in the median eminence (Herde et al., 2013).

GnRH bipolar morphology, with GnRH neurons exhibiting one axon and one dendrite has been questioned since researchers failed to detect axonal markers on both GnRH projections. Only a minority of adult GnRH neurons exhibit an axon-like projection without spines (Herde and Herbison, 2015). On the contrary, the great majority of adults GnRH neurons contain spines on both their axon and dendrite, suggesting that GnRH neurons have two dendrites (Herde and Herbison, 2015). However, the GnRH dendrites have a unique characteristic of initiating action potentials. Action potentials are identified through ankyrin G immunoreactivity, with nearly 40% of GnRH dendritic regions containing ankyrin G, and a higher number of GnRH neurons expressing ankyrin G in their process going towards the median eminence (Herde and Herbison, 2015). At the median eminence level, GnRH terminals are able to both receive information and propagate action potentials. For this reason, GnRH processes are referred to as "dendron" due to their dendritic and axonal properties (Herbison, 2015).

3.5 GnRH neuronal projections

Although they are mainly located in the median eminence, GnRH neuronal projections have been identified in several brain regions. Retrograde tracing studies suggest that 50-70% of GnRH neurons project to the median eminence in mice, with the remaining 30% not being hypophysiotropic (Herbison, 2015). Some of these extra-hypothalamic GnRH process present in cortical and hippocampal regions may also be hypophysiotropic as recently demonstrated in male mice (Manfredi-Lozano et al., 2022). In primates, similar studies in cynomolgus macaques revealed that only 40% of GnRH neurons project to the median eminence (Goldsmith et al., 1990). Non-hypophysiotropic projections have been described in the central nervous system, specifically in the amygdaloid complex, the stria terminalis, the habenula, the hippocampus, the interpeduncular nucleus, the neocortex, the periaqueductal gray, and all circumventricular organs, such as the organum vasculosum of the lamina terminalis (OVLT)

(Witkin and Silverman, 1983; Herbison, 2015). The functions of these non-hypophysiotropic projections remain unknown. However, they may indicate that GnRH serves other non-reproductive purposes (Goldsmith et al., 1990; Herbison, 2015).

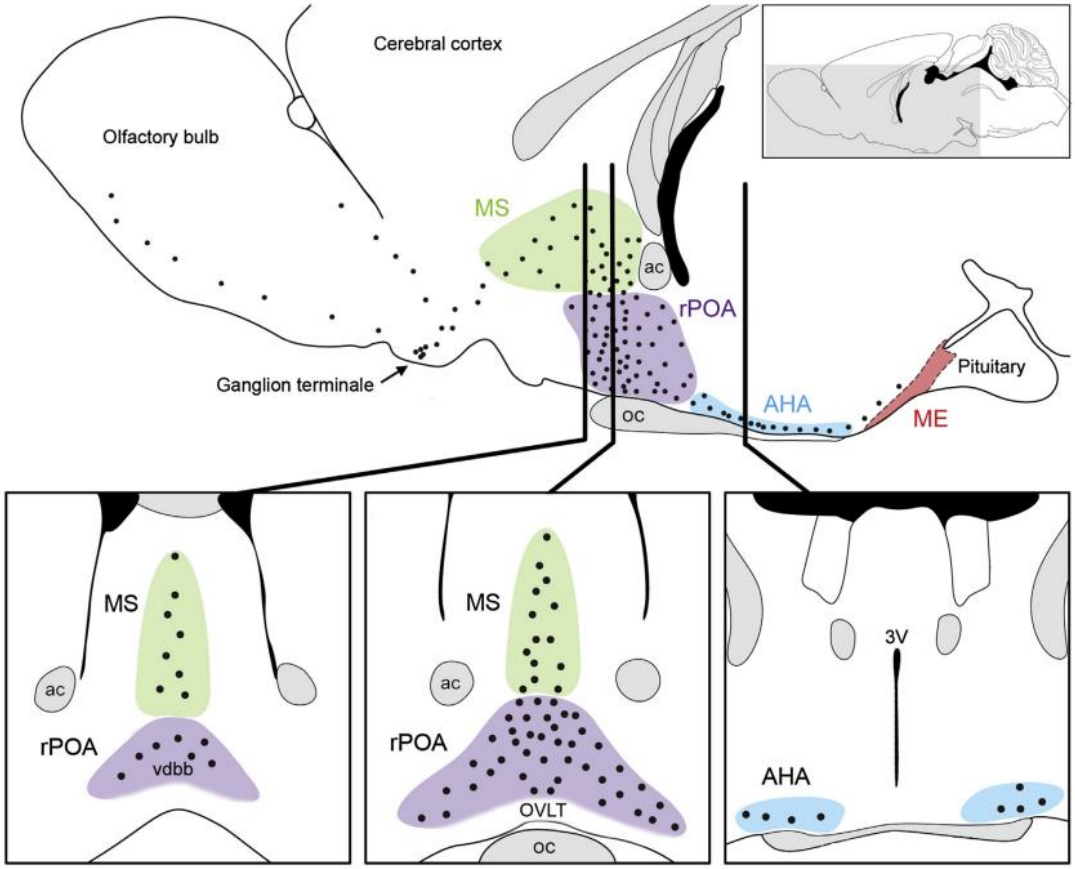

Figure 4: GnRH neuronal distribution in the mouse brain. Sagittal (top) and coronal (bottom) schematics show the distribution of GnRH neurons extending from the olfactory bulbs to the median eminence. GnRH cell bodies are mostly located in the medial septum (MS), the rostral preoptic area (rPOA), the anterior hypothalamic area (AHA) with fibers projecting to the median eminence (ME). Abbreviations: ac: anterior commissure; oc: optic chiasm; vdbb: vertical limb of the diagonal band of Broca; OVLT: organum vasculosum of the lamina terminals; 3V: third ventricle. Adapted from Herbison, 2015.

IV. Neuropeptides expressed by GnRH neurons

4.1 GnRH-I associated peptide

The GAP peptide, as previously mentioned, is located within the GnRH pro-hormone sequence and separated from the GnRH sequence by a cleavage site consisting of three amino acids. Through the use of antibodies in ovariectomized ewes, the presence of GAP was detected in both the GnRH nerve terminals and the hypophyseal-portal-system (Clarke and Cummins, 1987; Merchenthaler et al., 1989). GAP is secreted in the hypophyseal-portal-system following the same pulsatility as GnRH and LH (Clarke and Cummins, 1987). The functional significance of the GAP peptide remains largely unknown. Few studies conducted in the late 1980s probed into the physiological role of this peptide. Yu *et al.*, in 1988, provided evidence on the weak role of GAP promoting gonadotropin release but they observed that intravenous administration of GAP in ovariectomized (OVX) adult female rats actually suppressed prolactin release. In the same year, Chandrashekar *et al*. demonstrated that intracerebroventricular injection of GAP in adult male rats stimulated LH release after 60 minutes. Nonetheless, it did not affect the secretion of FSH, testosterone, or prolactin. The exact mechanisms behind these effects are yet to be determined, and further investigations are necessary to evaluate the role of GAP.

4.2 Galanin

GnRH neurons express both GnRH and galanin along with the galanin receptor in a subset of GnRH neurons located in the preoptic area ($GnRH^{POA}$ neurons) (Merchenthaler et al., 1990). In rats, a sexual dimorphism exists where females have more $GnRH^{POA}$ neurons co-expressing galanin than males (Merchenthaler et al., 1991). However, no sexual difference was observed in mice or sheep. In addition, galanin is not expressed by GnRH neurons in monkeys (Herbison, 2015). Galanin is secreted following GnRH pulsatile activity and facilitates the release of GnRH and LH in the median eminence through an autocrine action (Lopez and Negro-Vilar, 1990). Knockout mice lacking the galanin or galanin receptor in GnRH neurons do not eliminate GnRH or LH secretion. However, the LH surge in females is abolished upon i.c.v. injection of galantide, the galanin antagonist (Sahu et al., 1994). These findings suggest that galanin plays a role in the pre-ovulatory surge in rodents.

4.3 Glutamate

Glutamate and the vesicular glutamate transporter 2 (vGlut2) are expressed by GnRH neurons (Hrabovszky et al., 2004). The role of glutamate in GnRH neurons and GnRH release is unclear. Several studies suggest that glutamate facilitates GnRH release at the pituitary level. Moreover, glutamate may help GnRH neurons to self-regulate their excitability by inducing depolarization. Additional studies are required to confirm these findings. Currently, the role of glutamate in GnRH neurons remains to be determined (Iremonger et al., 2010).

V. Neuroendocrine control of reproduction

The onset of puberty and subsequently the activation of the HPG axis is preceded by two different pre-activations of the system. The first activation occurs during fetal life and the second activation, called mini-puberty, occurs during the first post-natal period in mice and humans. After these activations, the HPG axis remains quiescent until the onset of puberty (**Figure 5**). The mechanisms triggering mini-puberty, puberty, and the quiescence of the HPG axis are still not fully understood.

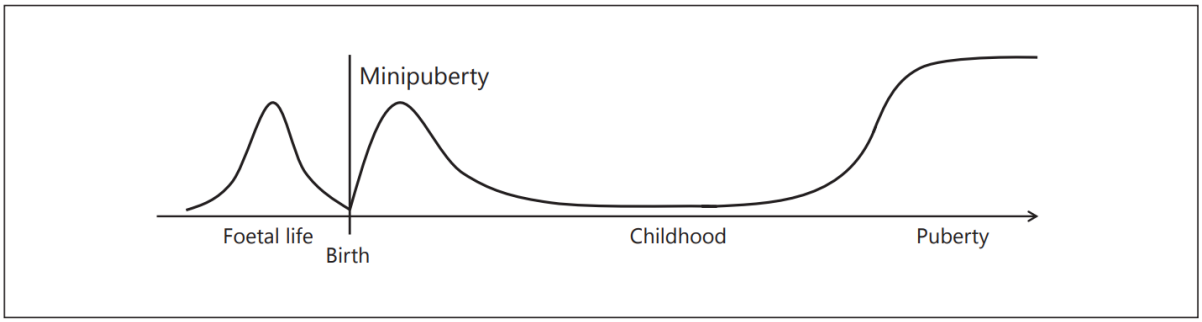

Figure 5: The three activations of the hypothalamic-pituitary axis in human. The activity of the HPG axis increases during fetal life, at mid-gestation and decreases during the last trimester, until birth. The second activation of the HPG axis happens around 1 week of age. The first post-natal gonadotropin surge, called mini-puberty, peaks at about 1 to 3 months of age. Then, the HPG axis activity remains quiescent until puberty (From Kuiri-Hänninen et al., 2014).

5.1 Pre-pubertal activation of the HPG axis

5.1.1 Fetal activation of the reproductive axis

While reproductive structures are in place at birth, further processes are required to achieve gonadal maturation, which commences during embryogenesis. Indeed, during human fetal development a transient activation of the reproductive axis takes place, characterized by an increase in secretion of LH and FSH. Then, during the last trimester of pregnancy in humans, the HPG axis activity decreases as a results of significant placental estrogen production. This transient activation may contribute to gonadal development later in life (Kuiri-Hänninen et al., 2014).

5.1.2 Mini-puberty

Mini-puberty is the first post-natal activation of the HPG axis. It occurs in humans between the first week of age and 3 months, and in mice around P10 to P15. During mini-puberty, gonadotropin levels are sexually dimorphic in humans: FSH levels are higher in girls than in boys, whereas LH levels predominate in boys. In boys, testosterone levels peak at 1-3 months and decrease at 6 months of age. Conversely, estradiol levels in girls remain elevated until 3-4 years old. This transient activity contributes to the maturation of the gonads in both males (testicular growth and testicular descent) and females (maturation of ovarian follicles) and is essential in achieving sexual maturity later in life. Finally, the HPG axis returns to its inactive state, through a mechanism which is still unknown, until the onset of puberty (Kuiri-Hänninen et al., 2014).

5.2 Puberty

Puberty corresponds to the transition from childhood to adulthood and is influenced by various genetic, environmental, and nutritional factors. Puberty occurs between 10 and 14 years old and is marked by the development of secondary sexual characteristics leading to sexual maturation. The reactivation of the HPG axis during puberty results in increased hormonal levels, causing changes in body composition, necessary to achieve sexual capacity. The maturation of the reproductive system induces breast development, apparition of pubic hair, genital development, menarche and spermarche (Abreu and Kaiser, 2016). The

reactivation of the system is directly linked to the activity of the GnRH neurons. The initiation of puberty occurs when GnRH neuronal firing and secretion increase due to removal of inhibitory factors and increase of excitatory inputs. Different actors play a role in the initiation of puberty, such as GABA, glutamate, kisspeptin or the neuropeptide Y, by increasing GnRH neuronal firing. In addition, puberty onset and fertility maintenance are influenced by fat reserves in the body. Evidence shows a correlation between metabolic status and the reproductive axis in individuals with excess or particularly low-fat mass levels, with associated decreased fertility (Elias and Purohit, 2013). A hormone, called leptin, plays a critical role in puberty onset. Indeed, leptin acts as a messenger, conveying metabolic information to the hypothalamus. Leptin is synthesized by the white adipose tissue, and its level is positively correlated with the amount of body fat. After food intake, leptin level increases, whereas it decreases after fasting (Tena-Sempere, 2013). Congenital leptin deficiency has revealed the close association between leptin and reproductive health in both mice and humans. Patients with this rare genetic disorder fail to enter puberty and have hypogonadotropic hypogonadism. However, administering exogenous leptin restores puberty onset and fertility (Garcia-Galiano et al., 2014).

All these factors influence puberty onset and the exact mechanisms triggering the initiation of puberty is still under investigation (Spaziani et al., 2021). To conclude on this part, the HPG axis undergoes maturation from early neonatal stages into pubertal development, during which proper secretion pattern is set throughout adult reproductive life.

VI. Neurobiology of the GnRH secretion

6.1 GnRH pulsatile secretion

It has been previously mentioned that a pulsatile GnRH secretion is critical for maintaining the proper pacing of the HPG axis. Inappropriate control of pulsatile GnRH release is implicated in the pathophysiology of common endocrine disorders such as polycystic ovary syndrome and functional hypothalamic amenorrhea. High GnRH/LH pulse secretion is a hallmark of polycystic ovary syndrome, whereas extremely low LH levels are associated with functional

hypothalamic amenorrhea. In women of reproductive age, inadequate function of the HPG axis leads to menstrual irregularities and infertility (Daniels and Berga, 1997; Reame et al., 1985; Coyle and Campbell, 2019).

Pulsatile delivery of GnRH was highlighted by studies performed in the late 1970s. *In vitro* experiments performed with pituitary fragments perfused with continuous GnRH did not promote LH pulses compared to pulsatile short-term GnRH stimulation (Osland et al., 1975). These findings corroborate the notion that pulsatile secretion of GnRH promotes pulsatile LH release. Moreover, the *in vitro* results have been validated *in vivo* across multiple species. In rhesus monkeys, portal blood sampling from the pituitary stalk was conducted with continuous and pulsatile GnRH administration. Findings showed that episodic GnRH release in the portal circulation is essential for the proper functioning of the HPG axis (Carmel et al., 1976; Belchetz and Plant, 1978). The factors responsible for pulsatile GnRH secretion have undergone extensive investigation. Initially, research focused on the intrinsic production of GnRH pulses.

GnRH neurons must coordinate their activity to release GnRH in a pulsatile manner in the median eminence. It has yet to be determined whether GnRH neurons can communicate with each other. However, cellular interactions between GnRH neurons have been observed within the baboon hypothalamus, raising the hypothesis that a direct communication between GnRH neurons could generate internal pulses and synchronize these neurons (Marshall and Goldsmith, 1980). The GT1-7 immortalized cell line was used *in vitro* to demonstrate that GnRH neurons spontaneously release GnRH synchronously within a cluster of interconnected cells. This suggests the presence of a functional neuronal network within the GnRH neurons (Hiruma et al., 1997). GT1-7 cells communicate through a cell-to-cell communication since electrical stimulations propagate between cells (Hiruma et al., 1997). The communication mechanisms amongst GnRH neurons have not been fully clarified yet. However, GnRH is not the sole peptide released by the GnRH neurons, which implies the possibility of communication taking place via other neuropeptides secreted by GnRH neurons. Despite *in vitro* data, no evidence has been discovered in favor of *in vivo* synchronization of GnRH neurons. Electrophysiological recordings have not detected any form of synchronization (Herbison, 2018).

Furthermore, GnRH dye-filing techniques have illustrated that both smooth and spiny GnRH neurons are present in mammalian brain. Prior to puberty, GnRH neurons appear smooth, but following the onset of puberty, there is an increase in the number of dendritic spines exhibited by GnRH neurons which amplifies during adulthood. A neuroanatomical remodeling of GnRH neurons which involves an increase in dendritic spines is taking place during the pubertal developmental phase, which may magnify and synchronize GnRH firing activity at puberty through either a direct communication between GnRH neurons or the involvement of other inputs on GnRH neurons (Campbell, 2007). The hypothesis regarding intrinsic pulse generation lacks *in vivo* validation, which led to the investigation of the involvement of extrinsic pulse generation. Afferent inputs can provide synchronized control of GnRH neurons.

6.2 GnRH neuronal network

The GnRH neuronal population integrates both internal and external information, including energy balance, stress, hormone levels, and circadian rhythms. However, the neuronal network regulating GnRH synchronization and GnRH neuronal activity was only recently defined (Campbell, 2007). GnRH neurons cannot impinge steroid hormones feedback information to properly regulate the functioning of the HPG axis since they do not express the required receptors. The control of GnRH neurons is exerted by intricate hypothalamic circuits composed of a vast network of primary afferent neurons relaying feedback information and metabolic/environmental signals to GnRH neurons. These circuits constitute the GnRH neuronal network (Campbell, 2007). The GnRH neuronal network is then the gate to regulate GnRH neuron activity and secretory function throughout the reproductive life (Herbison, 2016). Below, I will detail the list of neuronal cell types identified so far in this network and which finely tune GnRH neuronal activity.

6.2.1 Kisspeptin neurons

Idiopathic hypogonadotropic hypogonadism (IHH) is a rare disease characterized by impaired gonadotropic axis function. In 2003, two independent groups identified for the first time a novel mutation responsible for IHH. The mutation affects the gene encoding for GPR54, an orphan G protein-coupled receptor, and results in small testes, delayed vaginal opening,

absence of follicular maturation in both male and female mice and impairs puberty and reproductive function in humans (De Roux et al., 2003; Seminara et al., 2003). The data suggest that the GPR54 receptor plays a crucial role in the gonadotropic axis. The ligand of this receptor, the kisspeptin 1 protein (Kiss1), has been identified and several studies have established the importance of kisspeptin neurons in pubertal onset and GnRH/LH secretion (De Roux et al., 2003; Seminara et al., 2003; Gianetti and Seminara, 2008). Furthermore, the expression of GPR54 or kisspeptin receptor (Kiss1R) was detected in GnRH neurons (Messager et al., 2005). Based on the previous results, it was hypothesized that kisspeptin neurons directly modulate pulsatile GnRH secretion (De Roux et al., 2003; Messager et al., 2005; Xie et al., 2022).

Kisspeptin neurons are mainly located in the hypothalamus, more precisely in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC). Recently, a population of kisspeptin neurons has been discovered in the medial amygdala (Cravo et al., 2011). The kisspeptin neurons in the AVPV contribute to the GnRH/LH surge by interacting with GnRH neurons, both at the level of the cellular body and the processes nearby the ME. The kisspeptin neurons situated in the ARC solely make contact with GnRH neurons at the distal processes level (Herbison, 2018). Strong evidence shows that kisspeptin stimulates GnRH activity and release by binding with its receptor located on GnRH neurons and pointed out the role of kisspeptin neurons as one important component of the GnRH neuronal network, but do not explain the pulsatile GnRH release (Messager et al., 2005). Additional work demonstrated that kisspeptin neurons located in the arcuate nucleus produce and express, in addition to kisspeptin, other molecules crucial for reproduction: neurokinin B (NKB) and its NK3R receptor, as well as dynorphin and its kappa opioid receptor (KOR) (Navarro et al., 2009). In an autocrine manner, NKB acts on kisspeptin neurons to stimulate the production and release of kisspeptin and ultimately GnRH (Sandoval-Guzmán and E. Rance, 2004), whereas dynorphin inhibits the neuronal activity of kisspeptin neurons, decreasing kisspeptin secretion, culminating in the termination of GnRH secretion (Navarro et al., 2009; Weems et al., 2018). NKB and dynorphin present opposite actions in the control of kisspeptin secretion which shape the GnRH release, inducing a pulsatile GnRH secretion. These ARC neurons expressing kisspeptin, neurokinin B, and dynorphin A, were referred to as KNDy neurons, and it is now largely accepted that they play a key central role in the regulation of GnRH pulse

generation and subsequent tonic gonadotropin release that controls mammalian reproduction (Herbison, 2016). Additionally, KNDy neurons also express glutamate, which might be a critical synchronizing factor driving the GnRH pulsatile release (Han et al., 2023). Finally, to support the importance of kisspeptin neurons in generating GnRH pulsatility it has been shown that unlike GnRH neurons, the ARN Kiss neurons express the steroid receptors, that are crucial for receiving feedback information and regulating reproduction (Wintermantel et al., 2006; McQuillan et al., 2022).

Kisspeptin neurons do not appear to be the only neurons shaping the GnRH pulsatile release since dynorphin alteration has no impact on LH pulsatility, and dynorphin activation by optogenetic does not decrease the number of LH pulses in gonadectomized mice suggesting that additional players within the GnRH neuronal network must be involved to shape GnRH pulsatile activity (Mostari et al., 2013; Han et al., 2020).

6.2.2 Nitric oxide

Clasadonte et al., in 2008 showed that nitric oxide (NO) spontaneously decreases GnRH firing activity *in vivo* in both male and female mice regardless of the estrous cycle stage. Nitric oxide is a gaseous molecule produced by neuronal nitric oxide synthase (nNOS) neurons. nNOs knockout mice are infertile and exhibit a hypogonadotropic hypogonadism phenotype. This mouse line presents abnormal GnRH levels, resulting in low levels of gonadotropins. These findings support the involvement of NO in the control of the HPG axis (reviewed by Chachlaki and Prevot, 2020). nNOS neurons are found in many brain areas such as the rostral preoptic area where they are in close apposition and intermingled with GnRH neurons. In the median preoptic nucleus (MePO), GnRH neurons are surrounded by nNOS neurons (Delli et al., 2021). NO's role in the generation of the GnRH pulses have been investigated more recently. First, studies demonstrate that NO stimulates the exocytosis of GnRH and pulsatile release of LH, making NO a good candidate for regulating GnRH activity and contributing to the GnRH neuronal network (Rettori et al., 1993). More recently, results demonstrated that nNOS neurons inhibit GnRH neurons action potential bursts in order to synchronize GnRH neurons activity enabling a pulsatile secretion (Constantin et al., 2021). Thus, nNOS and kisspeptin neurons might act in synergy to initiate and terminate GnRH pulsatile activity.

6.2.3 Neurotransmitters

In addition to the kisspeptin and NO, neurotransmitters such as glutamate and γ-aminobutyric acid (GABA) play a critical role in the afferent control of GnRH neuron activity by inducing excitatory or inhibitory responses (Iremonger et al., 2010). A wild range of glutamatergic and GABAergic neurons express the Estrogen receptor alpha (ERα) essential for the retro-control of the HPG axis. Moreover, GnRH neurons express functional N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), $GABA_A$, and $GABA_B$ receptors (Hrabovszky et al., 2004; Moore et al., 2018).

Involvement of glutamate in the regulation of GnRH activity

Glutamate is the most prevalent excitatory neurotransmitter used by neurons in the central nervous system. Glutamate exerts its action through ionotropic and metabotropic receptors: AMPA, kainate and NDMA receptors. These receptors are located on the postsynaptic neurons and ionotropic receptors transmit a fast response, while metabotropic receptors mediate a slower response (Kew and Kemp, 2005). Glutamatergic synapses trigger excitatory activity in most neurons in the brain, GnRH neurons are no exception, as they are regulated by excitatory glutamatergic inputs. AMPA/kainate receptors predominate over NMDA receptors in GnRH neurons (Iremonger et al., 2010). The slow transition from smooth to spiny GnRH neurons around puberty onset suggests a glutamate role in the maturation of the HPG axis. It has been demonstrated that NMDA antagonist infusion delay puberty, while NMDA antagonist administration advance puberty hallmarks (review by Maffucci and Gore, 2009). Moreover, the synthesis of glutamate by GnRH neurons increases concomitantly with GnRH secretion. Finally, glutamate receptors are present throughout the hypothalamus, indicating that glutamate control of GnRH release can be trigger by a direct or an undirect mechanism. The exact nature of the glutamatergic neurons controlling GnRH neuronal activity has not yet been identified and further investigations should be performed to gain insights into the mechanisms regulating glutamatergic GnRH neurons activity.

Involvement of GABA in the regulation of GnRH activity

GABA serves as the primary inhibitor neurotransmitter in the adult brain and plays a critical role in brain development, where it exerts an excitatory function in some immature brain

regions. GABA action is mediated through ionotropic $GABA_A$ and $GABA_C$ receptors, and metabotropic $GABA_B$ receptors. GABA is the second main neurotransmitter implicated in the regulation of GnRH activity. Indeed, GnRH neurons receive direct GABAergic inputs from multiple neurons and express predominantly $GABA_A$ receptor subunits, which have been identified through both, qPCR and immunohistochemistry analysis (Moore et al., 2018). GABA modulates GnRH neuronal activity, although, the function of GABA as an excitatory or inhibitory neurotransmitter in GnRH neurons remains unclear. GnRH neurons possess a high internal chloride concentration even in adulthood, indicating that GnRH neuron's equilibrium potential stays near to the chloride ion equilibrium potential. Therefore, GABA action leads to the exit of chloride ions, which results in excitatory depolarizing responses. However, articles have shown that GABA inputs to GnRH neurons can be both excitatory and inhibitory. Han *et al*. in 2002 demonstrated that GABA inhibits the majority of GnRH mature neurons, while DeFazio in 2002 and Moenter in 2005 both highlighted the excitatory role of GABA on mature GnRH neurons (Herbison and Moenter, 2011). It is possible that depending on the location of GnRH neurons, GABA exerts either an excitatory or inhibitory action.

GABA participates in different stages of GnRH development and physiology. First, during embryogenesis, GnRH neuronal migration is dependent on GABA depolarization of GnRH neurons (Fueshko et al., 1998b; Wray, 2001). Then, GABA has been shown to be implicated in the onset of puberty, through inhibitory actions in the hypothalamus. Finally, the implication of GABA has been confirmed in the initiation of the GnRH/LH ovulatory surge in females (Herbison and Dyer, 1991).

6.2.4 Glial cells

GnRH neuronal activity is tightly regulated by excitatory and inhibitory neurons. Additionally, GnRH neurons have close interactions with glial cells, which are known to influence neuronal communications. Glial cells can modulate synaptic transmission by enwrapping the neuronal membrane at the synaptic button, thereby reducing the availability of neurotransmitters in the synapses. This synapse model, which contrasts with classical synapses involving only two neurons, is called the tripartite synapses integrating glial cells into the synaptic communication system (Haydon, 2001).

Tanycytes can be first mentioned as glial cells involved in the modulation of GnRH release. They are located in the first of the five layers composing the median eminence. Indeed, the first layer is the ependymal layer, placed at the ventral surface, which consists of specialized ependymal cells known as tanycytes that form the floor of the third ventricle. These cells are connected by tight junctions, creating a barrier that prevents high-molecular-weight molecules from diffusing. The second layer is the subependymal layer, which comprises cells with neural stem cell properties. The third layer is the fiber layer, which is composed of vasopressin and oxytocin nerves. The fourth layer is the reticular layer, mainly composed of neuronal fibers. The fifth layer is the external layer where axon terminals adjoin the portal vessels (Ojeda et al., 2008). GnRH axons that reach the ME are in proximity to tanycytes during the course of their trajectory towards portal circulation. In female rats, tanycytes exhibit structural plasticity during the estrous cycle, whereby tanycytic end-feet extend or retract under the influence of circulating sex-steroids hormones. This plasticity facilitates or impedes GnRH terminals to reach the portal vasculature (Prevot et al., 1999).

Another type of glial cell, the astrocytes, have their cell bodies and processes in close apposition with GnRH neurons (Sharif et al., 2013). Astrocytes secrete prostaglandin E2 (PGE2), which is a factor eliciting membrane depolarization of GnRH neurons, activating them. In addition, the inhibition of prostaglandin production by astrocytes impairs GnRH neuronal firing supporting the role of astrocytes in the regulation of GnRH neuronal activity (Clasadonte et al., 2011).

Finally, the identification of a novel communication mechanism, neuron-to-neuronal progenitor, shows that GnRH neurons are recruiting newborn astrocytes during infantile period using prostaglandin D_2 receptor DP1 signaling. This recruitment is essential to activate the HPG axis during puberty. Absence of newborn astrocytes recruitment during infancy impairs GnRH neuron maturation and functional activation, leading to a sexual maturation delay in mice (Pellegrino et al., 2021).

Altogether these results indicate that glial environment is essential to maintain the proper wiring and function of GnRH neurons.

VII. Factors affecting GnRH secretion

Reproductive function ensures the survival of the species and must adapt to environmental and physiological conditions. Seasonal breeders mainly rely on their circadian clock to regulate reproductive activity and avoid periods of drought, extreme heat, and ultimately food deprivation. Reproductive activity of nonseasonal breeders is based on their metabolic status and energy availability (Evans and Anderson, 2018). The hypothalamus and GnRH neurons integrate various information related to internal and external factors to regulate reproductive function. Coordination of stable energy homeostasis and external environment is essential for proper functioning of the HPG axis. In humans, obesity, stress, sleep disorder, shift work are known to impact reproductive function. For example, chronic energy deficiency, resulting in significant weight loss and strong fat mass decrease, such as what is observed during anorexia or in female athletes, leads to hypogonadotropic hypogonadism. Research indicates that an overnight fasting is enough to reduce LH secretion, supporting the hypothesis of a direct link between the two systems (Gamba and Pralong, 2006). Overnutrition and obesity have deleterious effects on spermatogenesis, sperm quality, and ovulation, ultimately impairing reproductive function (Barbagallo et al., 2021). The mechanisms coordinating the reproductive function in response to metabolic status are not fully understood but involve molecules such as the neuropeptide Y, leptin or insulin.

Messengers convey information regarding internal and external stimuli to the GnRH neurons or the GnRH neuronal network to coordinate reproduction. Among them, chemosensory inputs have been demonstrated to impact reproductive physiology and behavior in various species, including humans. Social interactions, and ultimately reproduction, are defined by olfactory cues, especially in rodents. Olfactory information found in urine have been shown to elicit changes in reproductive physiology and behavior in both male and female rodents. For instance, exposure to urine from a sexually active dominant male has been reported to accelerate sexual maturation in prepubertal females, a phenomenon known as the Vanderbergh effect (Vandenbergh, 1967). In a similar manner, male urine exposure induces estrus synchronization in female mice, this phenomenon is known as the Whitten effect (Whitten, 1959). Other olfactory information excreted by male rodents, and not only found in

male urine, cause another noteworthy phenomenon named the Bruce effect or pregnancy-block. This effect occurs when a female rodent, recently fertilized, is placed in the presence of another dominant male, resulting in a 70% pregnancy failure rate (Bruce, 1959). The physiological basis of the pregnancy-block effect has been characterized in detail. Olfactory information is collected by the nasal cavity and transmitted to the olfactory bulbs, and then to the amygdala and the hypothalamus. In the hypothalamus, an increase in dopamine secretion is observed in the ARC which leads to the inhibition of prolactin release at the pituitary level. Prolactin suppression causes regression of the corpora lutea, a fall in progesterone production by the ovaries, resulting in implantation failure (Brennan and Peele, 2003). All these reproductive changes have been reported in females. In males, exposure to opposite-sex smell promote neuroendocrine changes, such as a fast LH release (Maruniak and Bronson, 1976). The previously mentioned evidences suggest that GnRH neurons, in rodents, must receive either directly or indirectly, olfactory information to modulate reproductive physiology and behavior.

Other studies pointed out the link between olfactory information processing and reproduction in non-human primates. For example, it has been shown that primates employ olfactory cues to determine the sexual status of their conspecifics. A study, conducted by Michael and Keverne in 1968, tested whether two rhesus monkeys show a preference for an ovariectomized intravaginally estradiol-treated female or a non-estradiol primed rhesus monkey female. The study aimed to evaluate the monkeys' ability to discriminate the sexual status of the female subjects. To demonstrate their preference, monkeys were given access to the females by pressing a lever. Both males pressed the lever to gain access to ovariectomized females treated with intravaginal estradiol, but not the non-estradiol primed females. Then, the male monkeys were rendered anosmic via the insertion of gauze impregnated with bismuth-iodoform-paraffin into their nasal cavity, as well as through the cutting of the nerve supply to their Jacobson's organ. During this stage of the experiment, both males did not initiate lever pressing behavior specifically towards the ovariectomized estradiol-treated females. However, the restoration of their main olfactory functions proved sufficient to reinstate the lever pressing behavior for the estradiol-treated females (Michael and Keverne, 1968).

In humans, it was first reported that reproductive physiology impacts olfaction, especially in women. Olfactory sensitivity fluctuation during estrus cycle and pregnancy in women has been extensively investigated. Studies have shown that women have heightened olfactory sensitivity during the ovulation phase, with olfactory sensitivity decreasing during menses. Furthermore, during the ovulatory period, women perceive men's odors as more pleasant (Doty et al., 1981; Hummel et al., 1991; Pause et al., 1996). Olfaction also has an impact on human reproductive physiology. Studies have shown that opposite-sex smells have a greater effect on neuroendocrine and mood changes than same-sex smells. For instance, application of men's axillary extracts on the upper lip of women increases plasma LH level (Preti et al., 2003). However, Stern and McClintock reported that odorless compounds collected from women axillary regions during the late follicular phase of their cycle accelerate the onset of the preovulatory surge of LH and shorten the menstrual cycle of recipient women (Stern and McClintock, 1998). Some authors noted that not all literature supports these findings due to the effect's limited strength and detectability.

All this evidence indicates that in many species olfactory cues must modulate GnRH activity and secretion to coordinate and optimize reproduction. The following chapters will focus on the connections between the reproductive and the olfactory systems and the role of GnRH neurons in coordinating these systems.

VIII. References for chapter I

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Chapter 2: The olfactory system and reproduction

I. The sense of smell

Olfaction is one sensory modality highly conserved from invertebrates to mammals. The olfactory system can perceive, identify, discriminate and gather information facilitating decision-making leading to adapted behavioral responses. Information received through the olfactory system allows animals to detect numerous parameters and is crucial for finding food, defend territory, identify danger, for the recognition of conspecific, kin, predators or for the avoidance of toxic elements. The identification of chemicals signaling elicits stereotyped behavioral responses and play a role in mate choice, mother-infant recognition and signaling among members of a group. Hence, mammals rely on their sense of smell to guarantee the survival of the species and reproduction (Zarzo, 2007) .

To increase the chances of successful reproduction, animals synchronize their neuro-endocrine mechanisms with their breeding activities depending on social and environmental conditions. Among the social factors influencing the reproductive function, the sense of smell, or olfaction, stands out as perhaps the most influential. Olfactory cues, including pheromones, play a pivotal role in conveying species-specific information that influence reproductive physiology and function of individuals. The word “pheromone” comes from the Greek word *Pherin*, meaning to transfer, and from the Greek word *hormone*, meaning to excite. Pheromones are substance that can be excreted from an individual to the outside, received by a conspecific and act as a messenger between individuals (Dulac and Wagner, 2006). Pheromones have demonstrated their significant role as regulators throughout all phases of reproductive behavior, mating rituals, and parental care. In the context of sexual behavior, pheromones are indispensable for recognizing suitable mating partners, initiating subsequent sexual drive and copulatory activities (Keller et al., 2010).

Pheromones are distinct among species. Each species produces different pheromones, and in most cases, pheromones are only perceived by conspecifics. In addition, these non-volatile compounds are grouped into different categories such as: trace, territory, warning, maternal, or sexual pheromones. They are secreted by a lot of different types of glands and are found for instance in sweat, semen, vaginal secretion, urine, or saliva.

In humans, olfaction is more related to the quality of life, the selection and quality of food and plays a role in social interaction. Numerous pheromones’ effects on mood and cognition have

been reported. In humans, pheromones are also implicated in sexual attraction as it was described in the previous chapter (Doty et al., 1981; Hummel et al., 1991; Pause et al., 1996). Chemosensory cues are detected by two sensory epithelia, reach the forebrain center and then higher olfactory centers to mediate their effects. These circuits and systems arrangement are conserved throughout evolution in invertebrates and vertebrates, and they will be described below. Two additional subsystems, called the septal organ of Masera and the Gruenberg ganglion have also been described as involved in olfactory information processing, but little is known about their involvement in reproductive behavior. The Gruenberg ganglion detects low temperature and the warning pheromones triggering fear behavioral responses (Keller et al., 2010).

II. The main olfactory system

The main olfactory system (MOS) mostly perceives small volatile chemicals odorants inhaled during breathing. Olfactory cues are detected by receptors inserted on the ciliated dendrites of olfactory sensory neurons (OSN). These neurons are located in the main olfactory epithelium (MOE) located at the posterior part of the nasal cavity. In the MOE, OSN processes express olfactory receptors (ORs), which are seven transmembrane G-protein coupled-receptors encoded by around 1,000 genes in mice, 160 genes in zebrafish and around 350 genes in humans. Notably, OR expression is restricted to the epithelium (Buck and Axel, 1991), and each subtype of olfactory sensory neuron expresses only one type of olfactory receptor (Ressler et al., 1994). OSN expressing the same receptor project to and are gathered into glomeruli in the main olfactory bulb (MOB) and the glomeruli sharing common features are placed close to each other (Zarzo, 2007). Humans have approximately 15,500 glomeruli, mice nearly 1,800 and zebrafish 140 (Grabe and Sachse, 2018). In the glomeruli, OSNs make synapses with dendrites of mitral/tufted cells, each of these cells projects a unique dendrite to a specific glomerulus. This organization improves signal to noise ratio and increase the sensitivity to detect a specific stimulus (Zarzo, 2007). Thus, one single odorant is recognized by different olfactory receptors triggering the activation of different glomeruli constructing a coding path for odorant identification (**Figure 1**). Finally, olfactory information is transmitted to numerous forebrain areas such as: the olfactory tubercle (OT), the anterior olfactory

nucleus (AON), the tenia tecta (TT), the piriform cortex (PC), the entorhinal cortex (EC), the amygdala (**Figure 3**)(Dulac and Torello, 2003).

Another kind of olfactory receptor is located on the olfactory epithelium and are named the trace amine-associated receptors (TAARs) and detect ketones, acids and amines. On the contrary, OR detect acetates, aldehydes and aromatics. TAARs are expressed in unique subsets of OSN dispersed in the olfactory epithelium (Liberles and Buck, 2006). Three ligands for mouse's TAARs were identified in mouse urine. In mouse, TAAR4 recognizes a volatile amine, present in urine in response to stress; TAAR3 recognizes an isoamylamine found in male urine. This isoamylamine is known to accelerate the onset of puberty in female mice (Liberles and Buck, 2006). Finally, mouse TAAR5 is activated in presence of trimethylamine, a compound exclusively found in mature male mice urine (Liberles and Buck, 2006). Thus, TAARs may support the recognition of gender and sexual status of conspecifics in mice and may mediate physiological and behavioral responses upon social encounter.

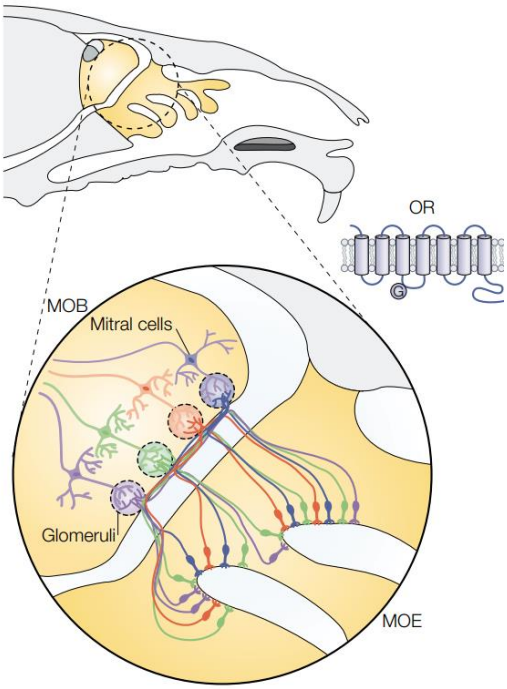

Figure 1: Odorants identification in the main olfactory system. Olfactory sensory neurons (OSN) express olfactory receptors (OR) on their dendrites inserted in the main olfactory epithelium (MOE). OSN expressing the same OR make synapses with mitral/tufted cells in the same glomeruli. This organization allow the detection and the identification of odorants. Adapted from Dulac and Torello, 2003.

III. The accessory olfactory system

The accessory olfactory system (AOS) perceives information through the vomeronasal organ (VNO). The VNO or Jacobson's organ was first described in 1813 by Ludvig Jacobson as a secretory organ. The VNO is a bilateral cylindrical-shape structure located at the basis of the nasal cavity, inside bone and along the nasal septum (Jennes, 1986). Non-volatile pheromones enter the VNO throughout direct contact in the nasal cavity, being pumped into this structure to serve as the first relay of pheromonal information. The dendritic terminals of VNO neurons detected the water-soluble molecules actively transported in the liquid-filled space of the VNO from the nasal cavity through dendritic microvilli. Within the VNO, two types of sensory neurons can be distinguished based on their neuroanatomical and molecular features. Sensory neurons located in the apical part of the VNO express one of the vomeronasal receptors belonging to the V1R family whereas sensory neurons located on the basal part of the VNO express one of the V2R family vomeronasal receptors. Although both receptor types are GPCRs, they are phylogenetically unrelated to each other and to the olfactory receptors, V1Rs share similarities with T2R family of bitter taste receptors, and V2Rs with metabotropic receptors exhibiting a very large N-terminal extracellular domain (Leinders-Zufall et al., 2000; Dulac and Wagner, 2006). V1R sensory neurons detect low molecular weight molecules, indeed, mice lacking of V1R failed to respond to small pheromones. Oppositely, V2R sensory neurons identify peptides due to the presence of a large hydrophobic amino-(N)-terminal extracellular domain (Dulac and Torello, 2003). Neurons expressing V1R and V2R pheromonal receptors project to the anterior and the posterior part of the accessory olfactory bulb (AOB), respectively, following a single axon accessing the AOB through the cribriform plate (**Figure 2**)(Dulac and Torello, 2003). Consequently, the information encoded by pheromones is transmitted to the AOB, located in the posterior dorsal region of the MOB, where sensory neurons gather in multiple glomeruli and synapse with the mitral cells of the AOB (Dulac and Torello, 2003).

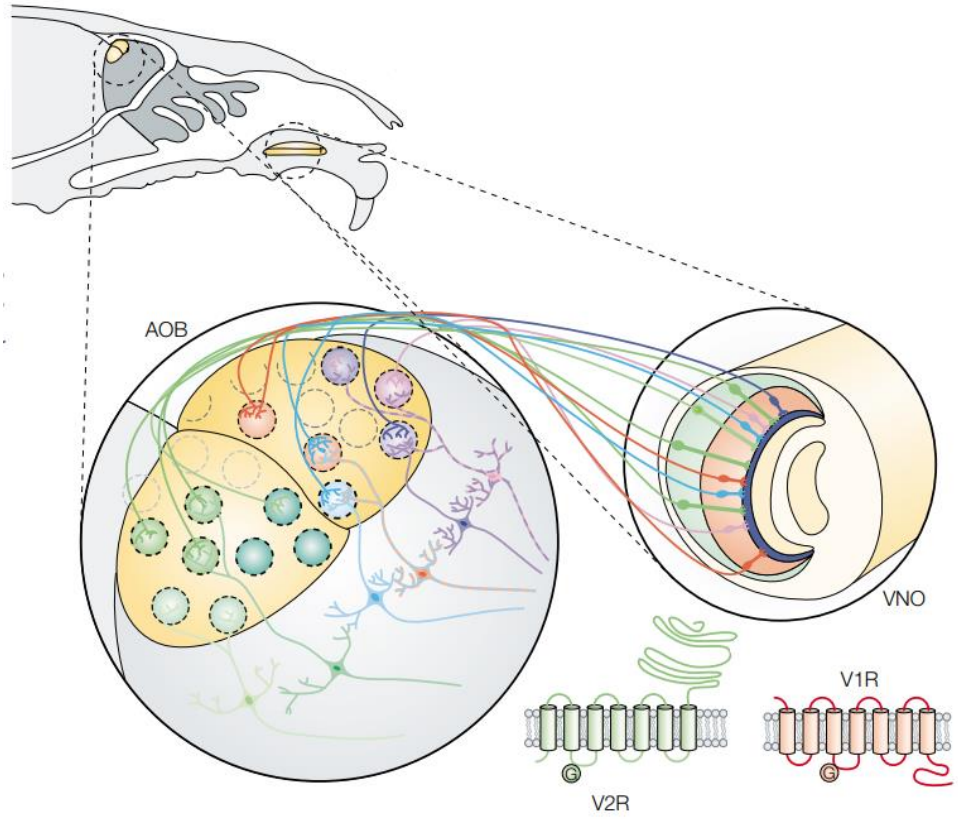

Figure 2: Odorants identification in the accessory olfactory system. The apical part of the VNO is composed of olfactory sensory neurons expressing the V1R pheromone receptors. In the posterior part of the VNO, OSN express the V2R pheromone receptors. Neurons expressing V1Rs are projecting to the anterior part of the AOB and neurons expressing V2Rs to the posterior part. In the AOB glomeruli, VNO sensory neurons make synapses with mitral/tufted cells. Adapted from Dulac and Torello, 2003.

Following a similar neuroanatomical pattern as the MOS, AOB glomeruli connected by neurons expressing closely related receptors of the same family gather in specific conserved positions of the AOB. In contrast with what was observed in the MOB, mitral cells in the AOB do not make synapses with only one glomerulus. In the AOB, mitral cells that project to different glomeruli innervated by sensory neurons expressing the same receptor subtype are described as homotypic (Luo, 2003; Dulac and Wagner, 2006). On the contrary, mitral cells that project to different glomeruli associated with different receptors exhibiting heterotypic connectivity (Dulac and Wagner, 2006). Most AOB mitral neurons project to the medial amygdala (MeA) with additional connections to the posterior bed nucleus of the stria terminalis (pBNST). The MeA transmits information to specific hypothalamic nuclei involved in the regulation of short-term innate behavioral responses and influence long-lasting neuroendocrine physiological responses. The MOS pathway is also indirectly connected to the hypothalamus, through its connections with the nucleus of the lateral olfactory tract (NLOT), the anterior cortical nucleus (ACN) and the posterolateral cortical amygdaloid nucleus (PLCN). (**Figure 3**). Within the hypothalamus, the medial preoptic area (MPOA) and the ventromedial nucleus of the hypothalamus (VMH) are involved in the regulation of reproductive, aggressive, parental behaviors and in the regulation of hormonal secretions, such as GnRH release. The connections between the MOS and the AOS with the hypothalamus could influence and orchestrate reproductive physiology and behavior, which is also a subject of the present study.

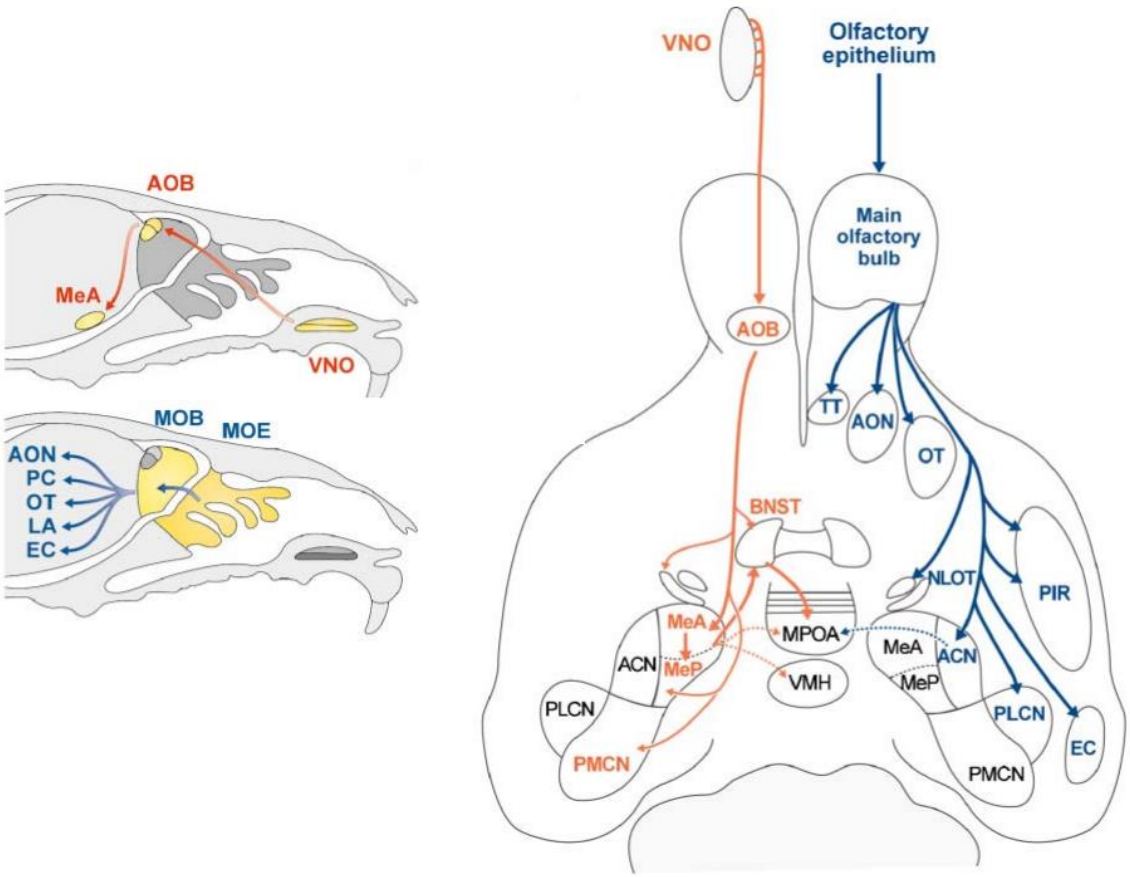

Figure 3: Two brain circuits relay olfactory information in mice. The main olfactory system gets its information from the main olfactory epithelium (MOE) which communicates with the main olfactory bulb (MOB). From the MOB, projections are sent to the anterior olfactory nucleus (AON), the piriform cortex (PC), the olfactory tubercle (OT), the lateral amygdala (LA) and the entorhinal cortex (EC). The vomeronasal system send projections to the accessory olfactory bulb (AOB), which then heavily projects to the medial amygdaloid nucleus (MeA) and more lightly to the bed nucleus of the stria terminalis (BNST), to the posterior medial amygdala (MeP) and the posterior-medial cortical epithelium (PMCN) of the amygdala. MeA, MeP and BNST directly project to the medial preoptic area (MPOA) of the hypothalamus. Figure adapted from Dulac and Wagner, 2006.

IV. The vomeronasal organ in human

Can humans detect pheromones? And, therefore, could pheromone detection impact human behavior and physiology? Evidence shows that humans are able to detect pheromones in different physiological states. For example, exposure of newborn infants to lactating women

secretion from the areolar glands of Montgomery induces, in these newborn infants, an automatic response of suckling, increases inspiratory activity and appetitive oral responses (Doucet et al., 2009). In adults, pheromones have been shown to elicit behavioral responses, such as ovulation synchronization in women and men attraction towards women during the ovulation phase (Doty et al., 1981; Hummel et al., 1991; Pause et al., 1996). However, the complete mechanisms behind these phenomena remain difficult since the VNO in human is known to degenerate at birth and no human pheromone have been purified. In humans, the VNO is located in forward position on the base of the septum near to the nasopalatine duct. The VNO develops *in utero*, as in rodents, vomeronasal nerves and GnRH neurons migrate from the medial part of the olfactory placode giving the future VNO towards the brain. However, after initial development, the VNO regresses and becomes vestigial. The histological structure of the VNO shows diverse signs of regression such as the absence of veins or turgescent tissue able to produce active pumping, the absence of epithelial receptor neurons and nerve fibers allowing to transport olfactory information (Trotier, 2011). In addition, the AOB structure is absent in higher primates, including humans. The AOB is detectable in human fetuses (Casoni et al., 2016), but as the VNO, regresses later in development.

These anatomical data were completed by genomic analyses, in which researchers tried to verify the presence of vomeronasal receptors in humans, similar to the vomeronasal receptors (V1R and V2R) discovered in rodents. However, all the genes encoding for vomeronasal receptors in humans are non-functional pseudogenes but five. These five genes were detected in the olfactory mucosa. The *trpc2* channel, essential to mediate pheromonal information in rodents, is also detected as encoded by pseudogenes. Can humans detect pheromones? The question remains. According to information mentioned previously, pheromones detection in humans does not seem to be possible. This interpretation contrasts with the results of numerous articles indicating that putative human pheromones elicit stereotyped physiological responses and affect the psychological state. However, whether pheromonal detection in humans could occur through the main olfactory system, rather than the VNO, is a fascinating hypothesis that remains to be investigated (Dulac and Torello, 2003; Trotier, 2011).

V. Synergistic role of the main and the accessory olfactory systems

The MOS and the AOS were considered as two independent systems, from both anatomical and functional points of view. The MOS was thought to be involved in the detection of a wide range of volatile olfactory cues, while the AOS identified conspecific information. The MOS and the AOS detect specific olfactory cues, and the activation of each circuit leads to diverse and specific behavioral output. More recently, studies offer contradictory findings supporting the idea of a synergistic role played by the two olfactory systems in the regulation of olfactory-innate-behaviors. The main and the accessory pathways are directly and indirectly communicating with each other at several levels. Firstly, the detection of volatile chemosignals by the main olfactory system activates the VNO pumping mechanism. This, in turn, leads to an increased detection of environmental olfactory cues (Meredith, 1998). Moreover, the AOB receives direct input from the MOB. However, projections from the AOB to the MOB have never been observed. While connections between the two pathways have been established, the location of these connections is still unclear. In addition to the previous anatomical findings, studies about information processing with the activation of specific areas demonstrated that the main olfactory system is also carrying subsets pheromonal responses and, on the contrary, the VNO detects some volatile olfactory cues (Dulac and Torello, 2003). For instance, it has been shown that exposure to urinary volatile odorants increase cFOS expression within the AOB. Moreover, after lesioning the MOE and exposing mice to urinary volatile odorants, no significant changes were observed in cFOS expression in the accessory olfactory bulb when compared to control animals exposed to saline (Martel and Baum, 2007). These findings suggest a synergic role of these systems in detecting olfactory cues that influence behavioral outputs. Since both the AOB and the MOB pathways demonstrate direct connections to distinct amygdala nuclei, the amygdala could be an area of communication between the two olfactory systems. Indeed, neurons in the medial and cortical amygdala show responsiveness to inputs from both the MOB and the AOB. These findings provide evidence of functional convergence between main and accessory systems within the amygdala (Licht and Meredith, 1987). In addition, the MPOA has also been identified as an indirect area of communication between the two systems (Dulac and Wagner, 2006). The preoptic area receives connections from the anterior cortical nucleus of the amygdala and from the medial

amygdala, which are connected to the MOB and the AOB, respectively, making the POA another site of convergence of both olfactory systems.

VI. Olfactory systems involvement in olfactory preference

The detection of opposite-sex olfactory cues may elicit motivation to seek socio-sexual partners. Some specific olfactory cues present in urine, vaginal secretion or sweat allow to identify a conspecific. The major urinary proteins (MUPs), found in urine, give an individual identity signature and as a consequence provide gender-related information as well as health / metabolic / reproductive status and trigger behavioral responses when they are detected (Zhou and Rui, 2010). Other olfactory cues elicit attractiveness for a partner. For example, the aphrodisin protein, firstly identify in female hamster vaginal secretion is detected by males and facilitates copulatory behavior (Briand et al., 2004). On the contrary, the (methylthio)methanethiol (MTMT), found in male urine, is a strong-smelling component attractive for females (Zhang et al., 2018). More recently, odor hedonics coding in the vertebrate olfactory bulb has been investigated and results demonstrated that the dorsal part of the OB encodes neutral hedonic value of odorants and those identified as dangerous. The optogenetic activation of this region induces freezing in mice. On the contrary, the ventral area of the OB encodes for appetitive and social odors. The anteroventral part detects pleasant odors, while the posteroventral part of the OB detects unpleasant odors. The ventral domain is mainly composed of mitral cells, making synapses in the olfactory tubercle – an area that encodes the hedonic value of odor (Kermen et al., 2021). This specific organization within the OB must be essential to mediate behavioral responses to odorants.

Olfactory preference is a crucial factor facilitating sexual behavior in rodents. This olfactory preference is triggered by odorants and pheromones found in urine from both males and females, as well as in vaginal secretions. Most male mice, regardless of their sexual experience, spend more time investigating female urine in comparison to male urine, indicating a preference toward opposite sex odors. Olfactory preference is mediated both by the MOS and the AOS pathways. Indeed, VNO ablation studies and the use of TPRC2 null mice (a cation channel essential to mediate pheromone evoked responses) show that in absence of

an intact VNO system, male mice do not show any preference (Beauchamp et al, 1985; Stowers et al., 2002). However, Pankevich et al., 2006 did not corroborate these observations and suggest that the VNO affects male mice motivation to investigate female smell while the VNO ablation does not abolish olfactory preference. Regarding the role of the MOS in the mediation of olfactory preference, lesioning studies applying intranasal zinc sulfate to the MOE showed that MOE lesion eliminates opposite-sex olfactory preference in mice (Keller et al., 2010). These results indicate that both olfactory systems are working in synergy to mediate olfactory preference by the detection of attracting opposite-sex olfactory cues, and by increasing the motivation to investigate odorants.

VII. Olfactory systems involvement in sexual behavior

Sexual behavior can be partitioned in two main phases: the appetitive phase and the consummatory phase. The role of the initiation phase is to elicit motivation using olfactory cues and pheromones to attract and detect a potential partner. The second phase allows copulation. In mammals, and more especially in rodents, olfactory cues detection facilitates sexual behavior. The coordination between the main and the accessory olfactory systems is crucial to maintain reproductive function. Indeed, VNO ablation in naïve male prairies voles impair copulatory behavior (Wekesa and Lepri, 1994). In the same way, VNO chemoreceptors removal in naïve male hamsters prevents mating behavior but has no effect on sexually experienced males (Meredith, 1986). On the contrary, TRPC2 null-mice display normal copulatory behavior (Stowers et al., 2002). VNO pathway might be important to facilitate copulatory behavior in naïve males, even if its contribution appears to vary according to species (Keller et al., 2010). Regarding the role of the MOE in copulatory behavior, numerous articles demonstrated that MOE lesions abolish sexual behavior in both sexually naïve and sexually experienced males (Keller et al., 2006; Yoon et al., 2005). Moreover, a complete bulbectomy in male rats disrupts the ejaculatory activity and the copulation effectiveness (Lumia et al., 1987). These results demonstrate that both olfactory systems are essential to control and maintain a reproductive behavior and function (Keverne, 2004).

Sexual behavior in males is dependent upon two areas: the BNST and the MPOA, which receive inputs from both the AOS and the MOS, respectively. The BNST has been shown to receive and send information to the AOB. A recent study shows that female urine triggers activation of the BNST, and more particularly, triggers activation of specific neurons expressing the estrogen alpha receptor (Bayless et al., 2023). Optogenetic and chemogenetic manipulations revealed that these neurons mediate sexual behavior and connect through GABAergic connections estrogen alpha receptor expressing neurons located in the MPOA. The MPOA is then connected to the periaqueductal grey (PAG) and to the ventral tegmental area (VTA), two centers that gate motor output and reward processing, respectively. Activation of the MPOA estrogen alpha receptor expressing neurons projections to the VTA and PAG increase the number of mounting and the number of intromissions. The VTA encodes for reward responses and send multiple projections to the nucleus accumbens (Nac), a reward hub. To evaluate whether MPOA estrogen alpha receptor expressing neurons activation induces dopaminergic secretion in the VTA and the Nac to encode reward responses, dopamine imaging was performed. Results demonstrated that activation of these neurons increase dopaminergic secretion in these areas, indicating a reward response during or after copulatory behavior (Bayless et al., 2023).

A sexual dimorphism exists in the brain regions regulating reproductive behavior. In female mice, lordosis behavior relies on pheromonal olfactory cues. In fact, VNO removal completely eliminates lordosis behavior in females (Hellier et al., 2018). Male urine exposure triggers activation of the BNST estrogen alpha receptor expressing neurons, as in male mice; however, a higher and longer activation have been observed in females (Bayless et al., 2023). In addition to the BNST, the rostral periventricular area of the third ventricle (RP3V) and the VMH have been reported to mediate lordosis behavior in female mice. Results demonstrated that the kisspeptin population located in the RP3V is activated after opposite-sex urine exposure and is an essential component of the neural network regulating lordosis behavior, working in synergy with the nNOS neurons of the VMH (Hellier et al., 2018).

Kisspeptin and nNOS neurons have been reported as being part of the GnRH neuronal network (Chapter 1), this is why the involvement of GnRH neurons in the modulation of sexual behavior has been investigated. Results demonstrated that sexual behavior, in females, is not

dependent on GnRH neurons (Hellier et al., 2018). In males, little is known about the role of GnRH in the modulation of reproductive behavior. Studies demonstrated that intracerebral GnRH injection is sufficient to restore copulatory behavior in naïve male mice following surgical VNO removal (Fernandez-Fewell and Meredith, 1994).

VIII. Olfactory systems involvement in aggressive behavior

Olfaction is the major sensory input regulating aggressive behavior. The olfactory system gives information about the intruder and allows the evaluation of the opponent's quality by sensing testosterone levels present in the opponent's urine for example (Guillot and Chapoutier, 1996). Indeed, olfactory cues present in male urine are detected when males are marking their territory. Urine contains pheromones eliciting inter-male aggression, such as the MUPs, and two volatile compounds the dihydroexobrevicomin and the 2-(s-butyl)-dihydrothiazole. Male mice with VNO ablation display reduced aggressive behavior, and genetic vomeronasal receptors ablation, as well as the use of TRPC2 null mice demonstrated an abolition of aggressive behavior, demonstrating the involvement of the AOS in male aggressive behavior (Takahashi and Miczek, 2013).

Downstream to the AOS system, several brain areas are involved in the mediation of aggressive behavior, among them: the medial amygdala, the prefrontal cortex (PFC), the MPOA, the lateral septum (LS), the VMH, the lateral hypothalamus (LatH), the BNST, the PAG and the locus coeruleus (LC). The hypothalamic area is the most-studied area in relation to aggressive behavior. Electrical and high intensity optogenetic stimulations of the VMH result in attack behavior in male rats and mice, showing that the hypothalamus triggers aggressive behavior (Lin et al., 2011). The prefrontal cortex is involved in decision making and its role in aggressive behavior have been reported in rodents and primates, including humans. The medial prefrontal cortex is activated when aggressive behavior is engaged and has an important inhibitory role in aggression. In addition, the PFC has connections to several brain areas involved in aggressive behavior such as the hypothalamus and the amygdala which makes it a central area in the regulation of this behavior (Takahashi and Miczek, 2013).

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Chapter 3: The olfactory GnRH system

I. Presence of GnRH neurons in the olfactory system

GnRH neurons located within the hypothalamus have been extensively studied and well described. However, as mentioned previously, GnRH neurons are not only located in the hypothalamus. In 1980, two articles described for the first time the presence of GnRH neurons in the olfactory system in adult hamster (Jennes and Stumpf, 1980; Phillips et al., 1980). In 1983, Witkin and Silverman reported the presence of GnRH neurons within the MOB, the AOB, the olfactory epithelium and the vomeronasal organ in adult rats. The presence of the olfactory GnRH system was confirmed in adult mice (Jennes, 1986), in the VNO and along the terminal nerve of adult hamster (Lehman et al., 1987). Within the VNO, GnRH processes do not contact other olfactory neurons, on the contrary GnRH processes end within the lamina propria of chemosensory mucosa (Wirsig-Wiechmann, 2001). Later, using the whole-mount immunolabelling and iDISCO clearing, Casoni et al. in 2016, confirmed previous results and the presence of an olfactory GnRH system in adult mice. They reported, that GnRH neurons are located within both the MOB and the AOB (**Figure 1**). Recent data from the laboratory show that the olfactory GnRH system is also present in adult human brains (**Figure 2**). Yet, the role of these olfactory GnRH neurons remains unknown. In the literature, data are only describing the presence of this system.

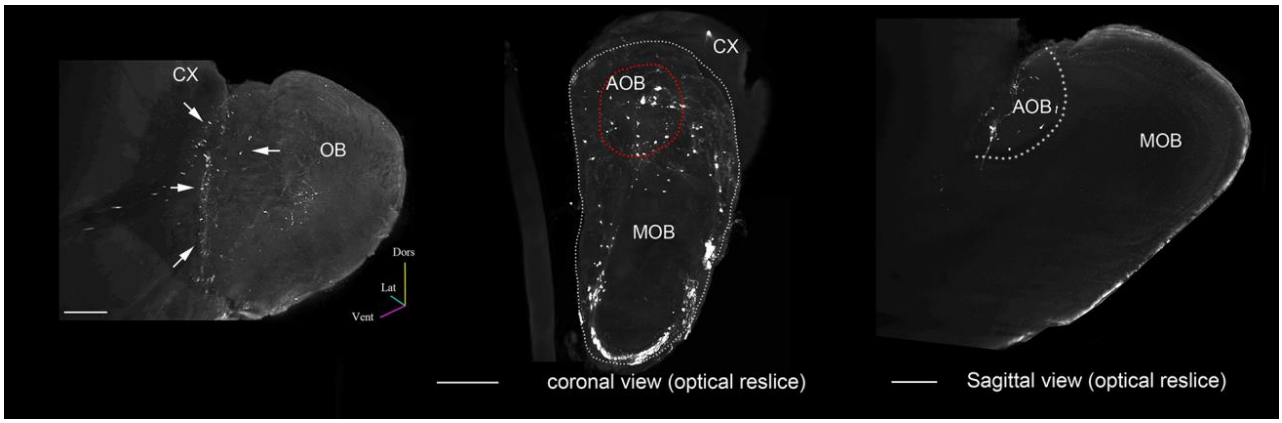

Figure 1: Whole-mount immunolabeling and iDISCO+ clearing of adult mouse brain. (A) Lateral view of the entire transparent olfactory bulbs (OB). (B) Coronal and (C) sagittal sections of the OB of adult male mice immunolabeled for GnRH. CX: cortex; OB: olfactory bulbs; AOB: accessory olfactory bulb; MOB: main olfactory bulb. Scale bars: A: 700µm; B: 500µm, C: 300µm. Adapted from Casoni et al., 2016.

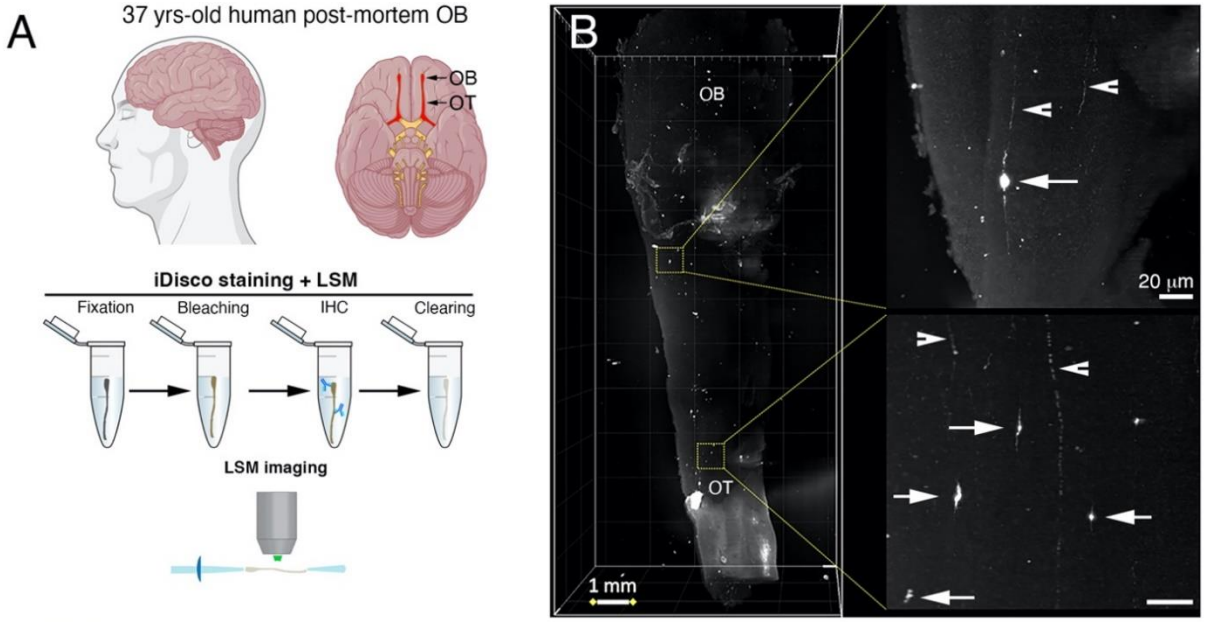

Figure 2: Whole-mount immunolabeling and iDISCO+ clearing of adult human OB. (A) Top: Lateral and inferior views of a human brain. The olfactory bulb (OB) and the olfactory tract (OT) are depicted in red. Bottom: schematic representation of solvent-based tissue clearing and light-sheet imaging protocol of the human post-mortem olfactory bulbs. (B) iDisco+ and light-sheet microscopy imaging showing the presence of GnRH neurons (arrows) and fibers (arrowheads) in the OB and OT of a 37-year-old postmortem brain (male). Representative pictures of N = 3 postmortem brains of men and women.

II. Presence of GnRH receptor throughout the olfactory system

2.1 The GnRH-R structure

The GnRH receptor (GnRH-R) is a member of the GPCR family. Receptors from the GPCR family have an extracellular N-terminal domain, a seven transmembrane segment and a cytoplasmic C-terminal domain. GnRH-R are part of the GPCR family, however, GnRH-R lacks of a cytoplasmic carboxy-terminal tail, which is not usual since this domain plays a crucial role in desensitization, internalization and signaling of the receptor. It was first admitted that the absence of the GnRH-R C-terminal domain was an evolutionary process exclusively found in

mammals but more recent studies demonstrated that the C-terminal domain of the GnRH-R was absent in many vertebrates (Flanagan and Manilall, 2017). Different subtypes of GnRH-R have been identified. The GnRH-R1 are tailless and the GnRH-R2 subtypes have a C-terminal domain. Few GnRH-R mutations were identified in human and lead to congenital hypogonadotropic hypogonadism (Costa et al., 2001).

2.2 Brain distribution of GnRH-R

GnRH neurons are not only projecting to the median eminence to release GnRH into the hypophyseal portal circulation. As mentioned in chapter I, diverse GnRH neuronal projections have been identified in widely spread brain areas. Studying the distribution of the GnRH-R throughout the brain allowed to gain insight about the role of GnRH as a neuropeptide. Since there are no specific antibodies directed against the GnRH-R, the creation of a transgenic mouse line (GRIC/R26-YFP) which expresses the green fluorescent protein (GFP) under the control of GnRH-R promoter facilitates the visualization of the GnRH-R expressing neurons (Wen et al., 2011). In the GRIC/R26-YFP male mice, GnRH-R are spread within the hypothalamus and the thalamus. In the hypothalamus, GnRH-R expressing cells are scattered through the preoptic area, the anterior, latero-anterior and dorsomedial hypothalamic areas. They were also identified in the arcuate and the periventricular nuclei. In the metencephalon and myelencephalon many GnRH-R expressing cells are found in the cerebellum and the pontine reticular nucleus while few are present in the raphe nucleus (Wen et al., 2011). Manfredi-Lozano et al., took advantage of the same mouse-line to show the expression of the GnRH-R in the cortex and the hippocampus. Unpublished data from the laboratory also showed the presence of GnRH-R expressing cells throughout the olfactory systems (**Figures 3 and 4**) and brain areas receiving olfactory information (**Figure 4**). Due to the presence of the GnRH-R expressing-cells in many different brain areas, it is tempting to speculate that GnRH may also play extra-reproductive functions, that I will describe in more detail in chapter 3.

2.3 Regulation of the GnRH-R expression

The absence of the C-terminal tail in the GnRH-R suggested an absence of internalization and renewal of the receptor. However, Maruska and Fernald, demonstrated that a modulation of

GnRH-R expression within the olfactory bulb is possible in *Astatotilapia burtoni* in response to environmental and internal physiological changes. Using qRT-PCR analysis, authors found that males express 2-3-fold higher levels of GnRH-R mRNA in the olfactory bulbs as compared to females (Maruska and Fernald, 2010). The mRNA expression levels of GnRH-R seems to be dependent on social status in males, with mRNA levels 3-fold higher in subordinate males than in dominant males, and on reproductive cycle and on reproductive state in females. Indeed, brooding or gravid females exhibit higher mRNA levels than other female individuals. Authors hypothesized that the elevated mRNA levels in subordinate males may reflect the necessity to detect more information in order to increase reproductive success (Maruska and Fernald, 2010).

Modulation of GnRH-R expression in response to environmental changes may be a potential mechanism explaining the behavioral and physiological neuroendocrine changes observed in response to olfactory cues. More investigations are required to confirm these results in mammals and to investigate the role of GnRH as a paracrine hormone in the extra-hypothalamic regions, such as the olfactory bulbs in olfactory-mediated reproduction.

Distribution of GnRH receptor expressing cells

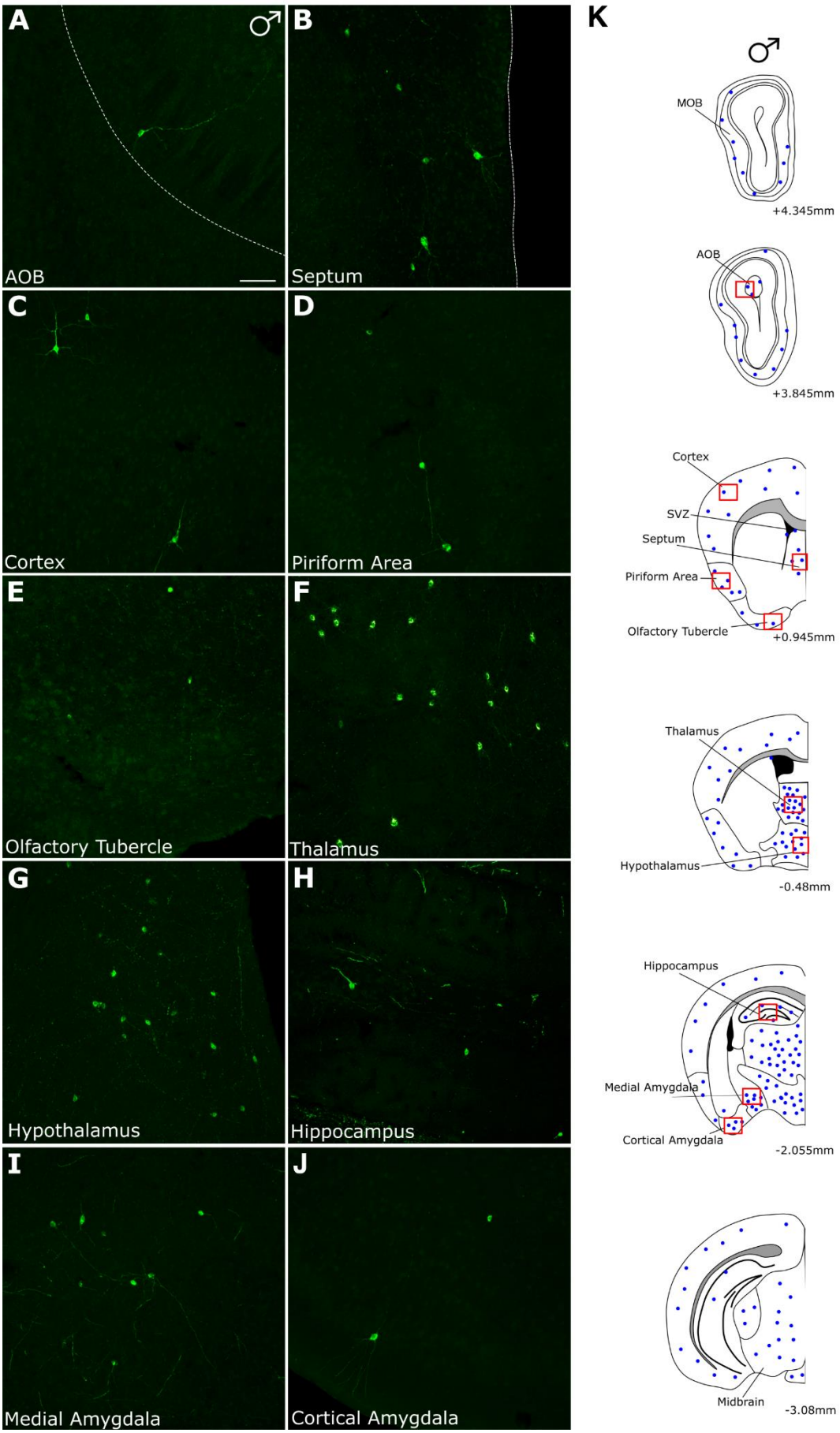

Figure 3: Distribution of GnRH-R expressing cells in the male mouse brain. Coronal sections and GFP immunostaining (green) reveal the presence of GnRHR expressing cells along different areas of the male mouse's brain: **(A)** AOB, **(B)** septum, **(C)** cortex, **(D)** pririform area, **(E)** olfactory tubercle, **(F)** thalamus, **(G)** hypothalamus, **(H)** hippocampus, **(I)** medial amygdala, **(J)** cortical amygdala. **(K)** Diagrams adapted from Paxinos and Franklin showing the representative distribution of GnRHR expressing cells in male mice brains. Lower number indicates the distance from bregma**.** AOB: accessory olfactory bulbs; MOB: main olfactory bulbs; SVZ: subventricular zone.

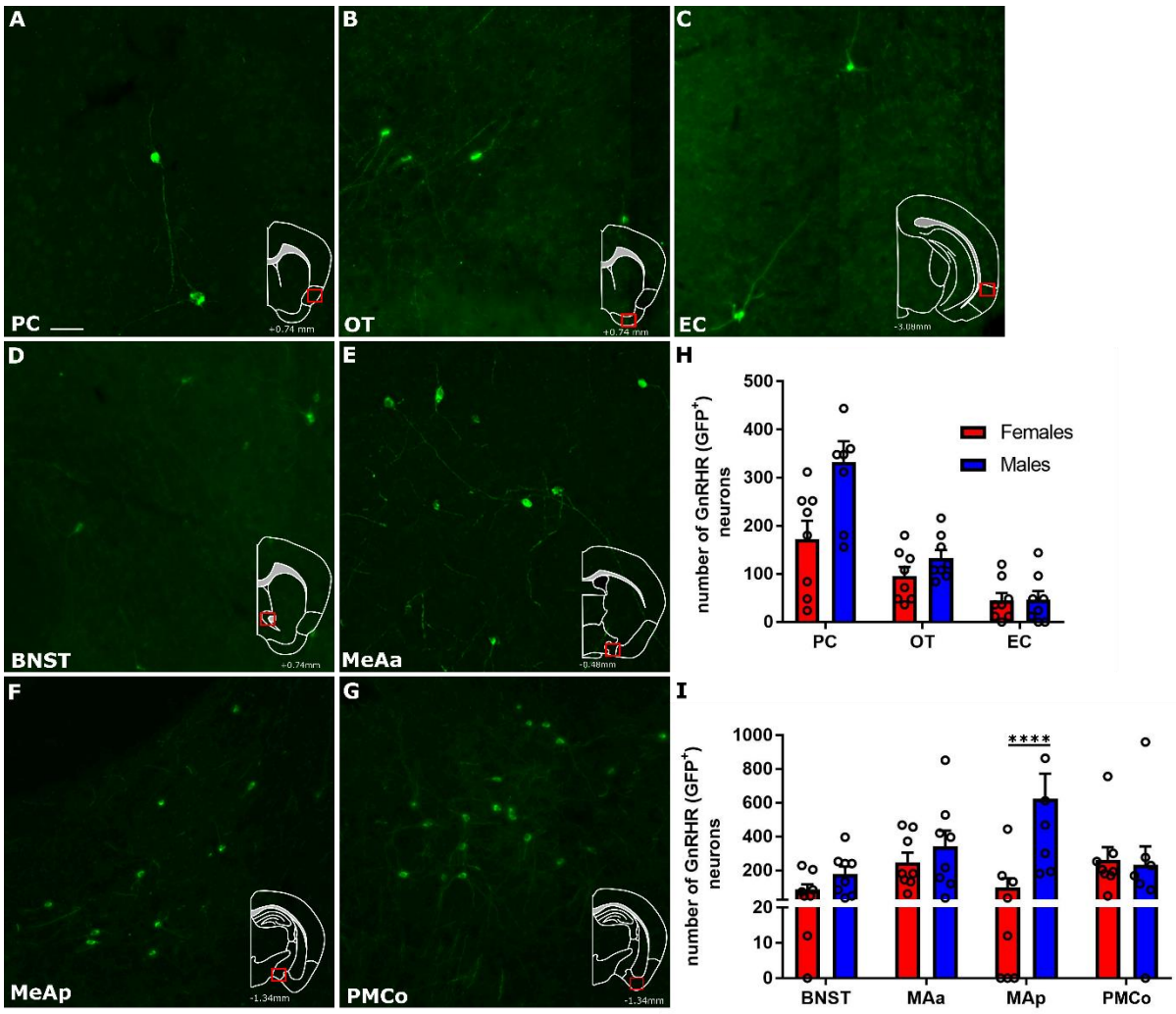

Figure 4: Distribution of GnRH-R expressing cells in selected brain areas. Coronal sections and GFP immunostaining (green) reveal the presence of GnRH-R expressing cells along **(A)** PC, **(B)** OT, **(C)** EC, **(D)** BNST, **(E)** MeAa, **(F)** MeAp, **(G)** PMCo, **(H)** Number of GnRH-R expressing cells in selected olfactory areas, **(I)** Number of GnRH-R expressing cells in selected forebrain areas. Lower number indicates the distance from bregma**.** PC: pririform cortex; OT: olfactory tubercle; EC: entorhinal cortex; BNST: bed nucleus of the stria terminalis; MeAa: anterior part of the medial amygdala; MeAp: posterior part of the medial amygdala; PMCo: posteromedial cortical amygdaloid nucleus.

III. Extra-hypothalamic roles of GnRH

GnRH has always been considered as a hormone that primarily regulates reproduction. However, as mentioned previously, GnRH receptors are widely expressed throughout the brain, where the GnRH signalling may exert multiple non-reproductive functions. In recent years, there's been an increasing interest in dissecting these extra-hypothalamic functions, which span from the regulation of cognition to the olfactory processing and mating behavior.

3.1 GnRH function in aging

GnRH pulse frequency gradually decreases with age in both males and females. Before this decrease, the frequency of GnRH pulses becomes irregular and, on the contrary, increases. The use of a chemogenetic approach allowed to reproduce this phenomenon and demonstrated that increased GnRH neurons firing activity leads to accelerate aging process in middle-aged male mice. Conversely, decreasing GnRH activity tended to slow down aging (Wang et al., 2021). This decline in GnRH secretion during aging may be attributable to an astrocytic dysfunction as they are highly sensitive to inflammation or to the immune pathway IKKβ/NF-κB (Wickramasuriya et al., 2022; Zhang et al., 2013). The immune pathway IKKβ/NF-κB in the hypothalamus in mice have been shown to be linked with aging process. Indeed, activation of the IKKβ/NF-κB increases aging process and diminishes lifespan. On the contrary, decreasing the IKKβ/NF-κB activity decreases aging process and increases lifespan. In addition, *Gnrh* mRNA levels are reduced with aging, and the inhibition or activation of the IKKβ/NF-κB pathway, increases or decreases *Gnrh* mRNA levels in mice, respectively, showing a link between this immune pathway, GnRH and aging. Then, it was observed that IKKβ/NF-κB activation reduces GnRH protomer activity. It was also demonstrated that GnRH administration through the third ventricle promotes adult neurogenesis in the hippocampus and in the hypothalamus, pointing out the central role of GnRH in aging process. The immune pathway IKKβ/NF-κB decreases GnRH activity, which in turn induces aging process of the hypothalamus by decreasing neurogenesis in this region. Exogenous GnRH administration or inhibition of inflammatory mediators decelerate aging progression in mice, highlighting the crucial role of GnRH during aging (Zhang et al., 2013).

3.2 GnRH role in cognition

Epidemiological evidences support GnRH's role in central functions as evidenced by a decrease in neurodegenerative diseases following treatment with GnRH agonist (Wickramasuriya et al., 2022). Indeed, Alzheimer's disease is marked by cognitive decline associated with memory deficits. In human, estrogen plays a major neuroprotective role in different areas including the hippocampus and the hypothalamus. As mentioned before, GnRH secretion is reduced with aging, impairing LH and FSH secretion. Evidence pointed out that elevated LH levels promote neurodegeneration by triggering cell death. However, the use of GnRH agonist or antagonist allows to regulate hormonal secretions and is associated with a lower cognitive decline (Wickramasuriya et al., 2022).

In addition, the role of GnRH in cognition has been recently reported in Down syndrome patients. Down syndrome or trisomy 21 is a common genetic disease associated with intellectual disability. Down syndrome patients present cognitive decline and deficit in sexual maturation. It has been shown in down syndrome mice model (Ts65Dn), which recapitulates many aspects of the human phenotype, that GnRH signaling progressively diminishes over the course of an individual's lifespan. The loss of GnRH corresponds to the age-deterioration of cognitive performances as well as olfactory deficit. In addition, in adult Ts65Dn mice extra-hypothalamic GnRH projections are absent, however, GnRH-R expressing cells are still present in extra-hypothalamic areas, such as in the cortex and the hippocampus. In the article, it was demonstrated that GnRH expression decreased because of an unbalanced production of different micro RNAs, in particular the miR-155 and miR-200 families, as well as an overexpression of *Zeb1* and *Cebpb*, two transcriptional repressor / activator genes. With the attempt of restoring cognitive functions, Ts65Dn male mice received a GnRH replacement therapy. GnRH pulsatile administration have been administrated first to mice, then to humans and improves cognitive functions, but partly restores the reproductive function in both mice and humans (Manfredi-Lozano et al., 2022).

To conclude, GnRH pulsatile secretion pattern alteration is observed with aging, impairing gonadotrophins and steroids hormones secretion. These impaired hormonal levels are associated with cognitive decline.

3.3 GnRH and olfaction

GnRH neurons are the major regulators of reproduction, and are mainly located within the preoptic area, which has been shown to integrate numerous various signals. Several reproductive neuroendocrine elements were depicted to be modulated in response to olfactory cues. For instance, Maruniak and Bronson, in 1976, showed that 15 minutes after exposure to female urine a fast and strong LH release is measured in male mice (**Figure 5**). They concluded that a factor contained in female urine must stimulate gonadotropin secretion in males. They also added that intact or castrated male urine, or female hamster urine were not able to elicit this LH secretion in male mice. The presence of GnRH neurons within the OB, and the connections between the POA and the olfactory systems suggest that these neurons may receive olfactory inputs to regulate reproductive physiology and behavior. Previous studies pointed out the connections between the AOS and the $GnRH^{POA}$ neurons, demonstrating that pheromonal signals activate these neurons (Fernandez-Fewell and Meredith, 1994; Meredith, 1998; Westberry and Meredith, 2003; Yoon et al., 2005). In addition, MOE lesion abolishes the activation of GnRH neurons after opposite sex-smell exposure but this was not observed in TRPC2 null-mice, meaning that the activation of GnRH neurons after opposite-sex smell exposure is induced by the connections with the MOS. In 2005, two articles showed for the first time, thanks to the use of pseudorabies virus and transneuronal tracers, that GnRH neurons located in the POA are receiving direct information from the olfactory bulbs. Articles noticed that these neurons have connections with the MOE and the VNO and with other areas involved in sexual behavior such as the amygdala, the PMCN, the BSNT, the ACN. GnRH neurons would act as integrator by receiving olfactory information and influencing diverse functions to optimize reproductive function (Boehm et al., 2005; Yoon et al., 2005).

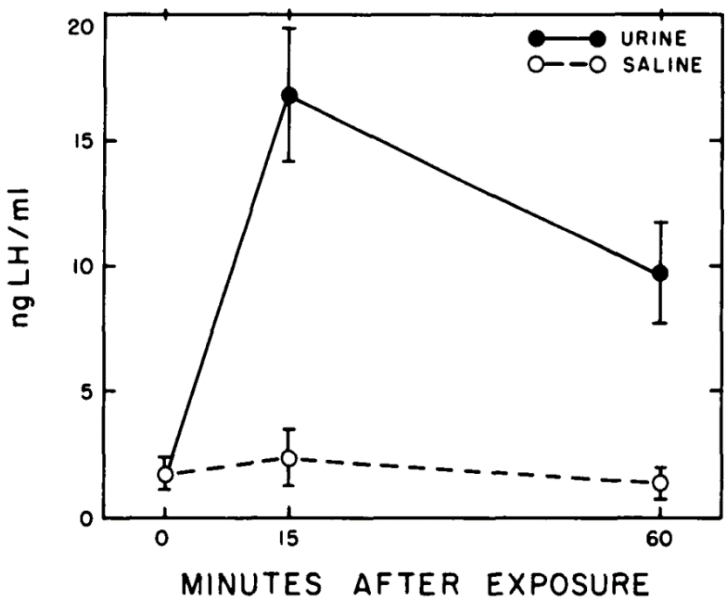

Figure 5: Exposure to female urine induces LH release in male mice. Female urine (pooled from intact, adult females from unknown stage of the cycle) effect on LH secretion in male mice (n=16 mice per point; vertical bars equal ± one SE). Adapted from Maruniak and Bronson, 1976.

These data show that GnRH neurons receive information from both olfactory pathways, implying that they could be able to modulate olfactory information processing. A mouse model abolishing GnRH expression after puberty (GnRH::Cre;Dicer$^{loxP/loxP}$) was used to evaluate the role of GnRH in olfactory preference (Messina et al., 2016). The lack of GnRH abolishes the olfactory preference in mice, however, an acute GnRH i.p. injection before performing the olfactory test restores the preference towards the opposite-sex smell, confirming the role of GnRH in mediating olfactory preference (Hellier et al., 2018). A further role for GnRH could be the coordination of olfaction and reproduction, however, neural circuits that mediate these effects remain unknown.

IV. Rational and objectives of the thesis

Altogether, the evidence provided above places GnRH neurons as a coordinating network, integrating olfactory information to facilitate reproductive function. Due to the presence of GnRH neurons within the olfactory bulbs and the presence of the GnRH-R along both olfactory systems we hypothesized that the olfactory GnRH system can be positioned as a coordinating system relying olfactory information to central brain areas to modulate reproductive physiology and behavior in response to olfactory cues.

Therefore, we set three objectives to our work: 1) investigate the anatomical distribution and transcriptomic profile of OB GnRH neurons; 2) determine the functional role of OB GnRH neurons; 3) examine the downstream targets of OB GnRH neurons.

To fulfill these objectives, we performed immunohistochemistry and took advantages of the iDISCO+ technique. To expand on the transcriptomic data, we performed single nuclei RNA sequencing. To figure out the functional role of OB GnRH neurons we performed stereotaxic injections of both activator and inhibitor DREADDs and used a combination of viruses to evaluate the requirement of OB GnRH neurons in the modulation of neuroendocrine and behavioral outputs in response to olfactory cues. Lastly, to put our observations into an integrative system and evaluate the role of OB GnRH neurons as a central regulatory hub we studied the downstream targets of OB GnRH neurons.

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Chapter 4: Material and methods

Animals

All animals were group-housed under specific pathogen-free conditions in a temperature-controlled room (21-22°C) with a 12-h light-dark cycle and *ad libitum* access to food and water. Animals were housed in individually ventilated cages, with a maximum of 6 animals per cage. Animal studies were approved by the Institutional Ethics Committees for the Care and Use of Experimental Animals of the University of Lille and the French Ministry of National Education, Higher Education and Research (APAFIS#2617- 2015110517317420 v5 and APAFIS#13387-2017122712209790 v9), the Universities of Turin and Homburg and by the National Council on Animal Care of the Italian Ministry of Health (authorization # 813/2018-PR) and the Department for Environment and Consumer Protection - Veterinary Section, Cologne, North Rhine-Westphalia, Germany. All experiments were performed in accordance with the guidelines for animal use specified by the European Union Council Directive of September 22, 2010 (2010/63/EU).

The sex, age and number of the animals used are specified in the text, figures and/or figure legends. Most male and female mice used in this study were sexually naïve; however, for the female odor-mediated LH release protocol, all male mice were sexually experienced following three confirmed successful breeding sessions. Investigators were blind to the genotype or treatment group of animals. Experiments were designed to minimize the number of animals used. C57Bl/6J GnRH::Cre (Tg(Gnrh1::cre)1Dlc)), *Gnrh::gfp* mice (Yoon et al., 2005) and C57Bl/6J GnRHR::IRES-Cre (GRIC)/eR26-τGFP (Wen et al., 2011) mouse lines were generous gifts of Dr. Catherine Dulac (Howard Hughes Medical Institute, Cambridge MA) and Dr Ulrich Boehm (University of Saarland School of Medicine, D-66421 Homburg, Germany).

iDISCO+

Head decalcification

When iDISCO+ was performed on intact mouse heads, decalcification of the samples was required prior to the pretreatment step. Decalcification was achieved by immersion of the heads in a solution of 20% disodium EDTA in PBS, for 2 x 3 days at room temperature with rotation. Samples were then rinsed in PBS and kept at 4°C until further processing.

Sample pretreatment with methanol

Samples were dehydrated in a methanol (MeOH) gradient: 40% MeOH, 60% MeOH, 80% MeOH, 100% MeOH, 100% MeOH in PBS for 1h each at RT on a rotating wheel, before overnight delipidation in a solution of 33% MeOH / 66% dichloromethane (DCM, Sigma-Aldrich) at 4°C. Next, samples were rinsed twice in 100% MeOH for 1h, and bleached in 5% H_2O_2 in MeOH at 4°C overnight. Finally, samples were washed in 100% MeOH for 1h and gradually rehydrated in 80% MeOH, 60% MeOH, 40% MeOH (1h each) and PBS (twice for 1h).

Whole-mount immunolabeling

Samples were incubated at RT on an adjustable rotator in a blocking solution of PBS containing 0.2% gelatin (Sigma), 0.5% Triton X-100 (Sigma-Aldrich) and 0.05% NaAzide (PBS-GT) for 4 days. Samples were transferred to a PBS-GT solution containing the following primary antibodies: anti-GnRH (Rat anti-GnRH #EH1044; dilution 1:10000; produced by Dr Erik Hrabovszky: Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine, Budapest, Hungary); anti-GFP (Chicken, dilution 1:5000, Aves Lab GFP-1020); anti-RFP (rabbit, dilution 1:1000, Rockland 800-656-7625), and placed at 37°C in rotation for 3 weeks. This was followed by several washes of 1h30 in PBS-GT at RT over two days. Next, samples were incubated with secondary antibodies (Goat anti-Rabbit AlexaFluor 488; Goat anti-Chicken AlexaFluor 568; Goat anti-Rat AlexaFluor 647; dilution 1:1000; Invitrogen) in PBS-GT and placed at 37°C in rotation for 10 days. Finally, samples were washed several times in 2 days for 1h30 each.

Tissue clearing

Samples were dehydrated in a methanol gradient: 40% MeOH, 60% MeOH, 80% MeOH, 100% MeOH, 100% MeOH in PBS for 1h each at RT on a rotating wheel and protected from the light. Delipidation was achieved with an overnight incubation in 33% MeOH / 66% DCM, and followed by two washes of 2h with 100% DCM. Samples were cleared in dibenzylether (DBE, Sigma-Aldrich) for 2h at RT with rotation and protected from the light. Finally, samples were moved into fresh DBE and stored in glass tubes in the dark and at RT until imaging.

3D imaging and analysis

3D Imaging was performed as previously described (Belle *et al.*, 2017; Casoni *et al.*, 2016) on the Ultramicroscope I (LaVision BioTec) equipped with a 1.1×/0.1NA and a 4×/0.3NA objectives and an Andor Neo 5.5 sCMOS camera. The light sheet was generated by a laser (wavelength 488nm, 568 nm or 647 nm, Coherent Sapphire Laser, LaVision BioTec) and two cylindrical lenses. Samples were placed in an imaging reservoir made of 100% quartz (LaVision BioTec) filled with DBE and illuminated from the side by the laser light. ImspectorPro software (LaVision BioTec) was used for image acquisition: the z-step between each image was fixed at 4 µm, and for tile imaging the overlap was set to 10%. The resulting sequences of tiff files were processed with Imaris Converter and Imaris Stitcher (Oxford Instruments), and Imaris 9.8 (Oxford Instruments) was used for visualization and analysis.

Tissue preparation for immunohistochemistry

Mice were deeply anesthetized via an intraperitoneal injection of a 3:1 ketamine (50-100 mg/kg) and xylazine (5-10 mg/kg) solution. All the animals were transcardially perfused with a 0.9% saline solution followed by cold 4% paraformaldehyde (PFA) in 0.05 M Tris-Buffered Saline (TBS), pH 7.4. Brains were removed from the skull and post-fixed for 4–6 h in 4% PFA at 4 °C. Post-fixing was followed by a cryopreservation step with a 30% sucrose solution in 0.05 M TBS pH 7.4 at 4 °C. Brains were sectioned by a microtome (Leica microtome). Free-floating coronal sections (35 µm) were collected in multi-well dishes to provide representative series of the brain. The sections were stored at −20 °C in an antifreeze solution (30% ethylene glycol, 20% glycerol, 50% 0.05M TBS; pH 7.4) until use.

Immunohistochemistry

Sections were rinsed in 0.05 M TBS; pH7.4; to remove the antifreeze solution and incubated in a blocking solution (0.05 M TBS; 0.3% Triton-X100; 0.25% Bovine-serum-albumin; 2% Normal Donkey Serum (NDS) for 1h at room temperature (RT). Then sections were incubated in a solution containing 0.05 M TBS, 0.3% Triton-X100, 1:100 of normal donkey serum and primary antibodies for 48h at 4°C. Section were rinsed in 0.05 M TBS; pH7.4; and then incubated in the incubation solution with appropriate fluorochrome-conjugated secondary antibodies, for 2h at room temperature. The following primary antibodies were used: anti-GnRH (rat; dilution 1:10000; produced by Dr Erik Hrabovszky: Laboratory of Endocrine

Neurobiology, Institute of Experimental Medicine, Budapest, Hungary; #1044); anti-GnRH (rabbit polyclonal antibody; dilution 1:2000; Proteintech; CatNo 26950-1-AP); anti-RFP (rabbit, dilution 1:1000; Rockland antibody mouse and rat serum proteins 800-656-7625); anti-GFP (chicken; dilution 1:1000; Aves-Lab GFP-1020); anti-cFos (mouse, dilution 1:500, Genetex GTX 60996); progesterone receptor (rabbit, dilution 1:500; Sigma SAB4502184); androgen receptor (rabbit, dilution 1:100; cell signaling #3202). Secondary antibodies were used as follow: Alexa Fluor 488 (1:500); Alexa Fluor 568 (1:500); Alexa Fluor 594 (1:500); Alexa Fluor 647 (1:500). Finally, sections were coverslipped with Invitrogen, Fluoromount-G™ with DAPI (REF: 00-4959-52), as an antifade mounting medium.

Cell counting

GnRH cell counts in the olfactory bulbs were performed on 35 µm coronal sections in an antero-posterior distribution. For each animal, cell counts were obtained using 1 out of 4 series of sections and multiplied by 4. GnRH neurons counts were performed by eye directly using an Axio Imager Z2 ApoTome microscope equipped with a motorized stage (Zeiss, Germany) and an ORCA-Flash 4.0 V2 camera (Hamamatsu, Japan) driven by the Zen imaging software (Zeiss) to account for the deepness of the tissue. Cell counting was performed by an investigator blind to the genotype of the animals.

Image acquisition and analysis

ImageJ (National Institutes of Health, Bethesda, MD) and Photoshop CS5 (Adobe Systems, San Jose, CA) were used to process, quantify, adjust, and merge the photomontages. Figures were prepared using Adobe Photoshop (Adobe Systems, San Jose, CA, RRID:SCR_014199) and Adobe Illustrator CS6.

snRNA sequencing

Tissue collection

The olfactory bulbs of 18 weeks old male C57Bl/6N mice (Charles River) were microdissected and immediately frozen in liquid nitrogen. Two biological replicates were analyzed, and each replicate comprised three olfactory bulbs.

Tissue homogenization and fluorescence-activated cell sorting (FACS)

Tissue homogenization was carried out in a 7 ml Dounce tissue grinder. Frozen olfactory bulbs were transferred into 1 ml of ice-cold homogenization buffer (NIM 2), IGEPAL 0.4% (v/v), RNasIn 0.4 μl^{-1}, SuperAsin 0.2 μl^{-1}, 0.15 mM spermine, 0.5 mM spermidine). Homogenization was carried out by 10 strokes of loose pestle 'A' followed by 20 strokes of tight pestle 'B' after a 5 min incubation on ice. Homogenates were filtered through 20 µm cell strainers and centrifuged at 1000 g for 10 min at 4°C. Supernatant was removed by decanting and the nuclei pellets were resuspended in 1 ml FACS buffer (0.15 mM spermine, 0.5 mM spermidine, 0.2 U μl^{-1} protector RNase inhibitor, IGEPAL 0,4% (v/v), BSA 1% (wt/vol) and DAPI 1µM in RNAase-free PBS). 300 µl of suspension from each of three individual nuclear isolations were combined for one replicate and subjected to FACS. Cell-sorting was performed with FACSAria III system (BD Biosciences) using a 70 µm nozzle. Gating for forward and side scatter was used for doublet discrimination and sorting was performed selecting singlets in the $DAPI^+$ population. 100.000 events were directly sorted into 10 µl of diluted nuclei buffer reaching a final concentration of ~1% BSA (wt/vol) and 0.2 U μl^{-1} protector RNase inhibitor.

Single-nucleus RNA-seq: RNA preparation, library generation, sequencing and analysis

Libraries were prepared using the Chromium Next GEM Automated Single Cell 3' Reagent Kit v3.1 (10x Genomics, #1000268) following the manufacturer's instructions. Libraries were sequenced on a HiSeq4000 (Illumina) with 150-bp paired-end sequencing of read 2.

A total of 13163 cells from two different sequencing runs were analyzed, comprising 1.02 billion total reads for an average read count of 77,489 per cell. Raw reads were processed with cell ranger (v.6.1.2) and aligned to Ensembl genome GRCm39 release 105_cr6.1.2 (Zheng et al., 2017). Approximately 92.4% of reads mapped confidently to the genome. All cells with at least 200 detected genes and 500 UMI were retained. Datasets were further merged and analyzed with Seurat (v4.1.1) (Hao et al., 2021) and cells with mitochondrial genes greater than 5% were discarded. Cells with at least one read for *Gnrh1* were annotated as *Gnrh* positive cells, which totaled 54 cells or about 0.4% of total cells. Read counts were normalized by a scaling factor of 10,000 followed by log transformation. The data was then scaled and centered using the ScaleData function with default parameters. A principal component analysis was performed and all dimensions where the percent change in variation against the next subsequent principal component was greater than 0.1% were used to construct a nearest

neighbor graph using the FindNeighbors function. Cells were clustered with a resolution of 0.2 and visualized by Uniform Manifold Approximation and Projection (UMAP). Clusters were manually annotated using FindAllMarkers functions to determine each cluster's significant marker genes. Markers were used to annotate clusters by matching them to known features of olfactory bulb populations (Imamura et al., 2020; Tepe et al., 2018). Some clusters were further subclustered using the FindSubCluster function if their expression profile matched known neuronal subtypes from literature. One cluster containing a small amount of contaminating Olfactory sensory neurons was manually removed as quality control. Go term analysis was performed using EnrichR with the GO_Molecular_Function_2021 and GO_Biological_Process_2021 libraries using the Seurat DEenrichrPlot function with default settings (Kuleshov et al., 2016). All visualizations were created using the Seurat package with modifications performed using ggplot2 (v.3.3.6).

Code Availability

All codes used in the analysis of data and visualization are available upon request.

Data availability

Files containing raw data and mapped reads will be uploaded to Gene expression omnibus and will be publicly available.

Fluorescence-activated cell-sorting (FACS)

The olfactory bulbs and the preoptic area of *Gnrh::Gfp* mice were microdissected and enzymatically dissociated using papain to obtain single-cell suspensions. FACS was performed using a SONY SH800 Sorter Cytometer device. The sort decision was based on measurements of GFP fluorescence (excitation: 488 nm, 50mW; detection: GFP bandpass 530/30 nm, autofluorescence bandpass 695/40 nm) by comparing cell suspensions from *Gnrh::Gfp* cortex. For each animal, 100 to 300 cells were sorted directly into 10µl of extraction buffer: 0.1% Triton-X100 (Sigma-Aldrich) and 0.4 unit/µl RNase OUT (Life Technologies).

Quantitative RT-PCR analyses

For gene expression analyses, mRNAs obtained from FACS-sorted GnRH neurons were reverse transcribed using SuperScript® III Reverse Transcriptase (Life Technologies) and a linear preamplification step was performed using the Taqman® PreAmp Master Mix Kit protocol (P/N 4366128, Applied Biosystems). Real-time PCR was carried out on Applied Biosystems

7900HT Fast Real-Time PCR System using exon-boundary-specific TaqMan® Gene Expression Assays (Applied Biosystems): *Gad1* (Gad1-Mm04207432_g1); *Gad2* (Gad2-Mm00484623_m1); *Gnrh1* (Gnrh1-Mm01315605_m1); *Grin1* (Grin1-Mm00433790_m1); *Grin2b* (Grin2b-Mm00433820_m1); *Grin3a* (Grin3a-Mm01341723_m1); *Slc17a6* (Slc17a6-Mm00499876_m1); *Slc17a7* (Slc17a7-Mm00812886_m1). Control housekeeping genes: *r18S* (r18S-Mm3928990_g1) and *Actb* (Actb-Mm00607939_s1). Gene expression data were analyzed using SDS 2.4.1 and Data Assist 3.0.1 software with R18S and actin as control housekeeping mRNAs.

RNAscope fluorescence *in situ* hybridization (FISH)

FISH was performed on 14 µm fresh frozen sections of the OB and the VNO of adult male mice using the RNAscope® Multiplex Fluorescent Kit v2 according to the manufacturer's protocol (Advanced Cell Diagnostics). Specific probes were used to detect *Gnrh1* (476281, XM_ 006518564.3, target region 81-914); *Vmn2r1* (1218801, NM_001384924.1, target region 150-2986); *Olfr323* (1218821, NM_146376.2, target region 2-958); androgen receptor (11835, NM_013476.3, target region 1432 – 2422); progesterone receptor (18667, NM_008829.2, target region 2695-3828); estrogen receptor α (26379; NM_007953.2, target region 2-769); kisspeptin (Kiss1, 280287; NM_178260.3; target region 5-485); GnRH-R (14715, NM_010323.2, 2-1099) mRNAs. Hybridization with a probe against the *Bacillus subtilis* dihydrodipicolinate reductase (*dapB*) gene (320871) was used as a negative control.

Electrophysiology

Brain slice preparation

Electrophysiological recordings were performed on living brain slices from 8 to 12-week-old mice. Mice were put under isoflurane anesthesia and killed by decapitation. The brain was dissected and rapidly placed in ice-cold aCSF containing: 120 mM NaCl, 3.2 mM KCl, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose (300 mOsm, pH 7.4) and bubbled with 95% O_2 to 5% CO_2. 200 µm sagittal slices containing the olfactory bulbs and the rostral preoptic area were cut using a VT1200 vibratome (Leica). Slices were incubated at 34°C in oxygenated aCSF for a recovery period of 1 hour, and then placed at room temperature until patch-clamp recording.

Patch-clamp recording

Individual brain slices were placed in a submerged recording chamber (Warner Instruments) and continuously perfused at a rate of 3mL/min with oxygenated aCSF maintained at 32.8°C by a heater controller (TC-344C-Warner Instrument). GnRH neurons were visualized under x10 and x40 magnification using an upright fluorescence microscope with infrared differential interference contrast (DM-LFSA, Leica) and an ORCA-Frash4.0 digital CMOS camera (Hamamatsu). Recording pipettes were pulled from borosilicate glass capillaries (1.5 mm outer diameter, 1.12 mm inner diameter; World Precision Instruments) using a P1000 Flaming Brown puller (Sutter Instruments) and had a resistance of 7-9 MΩ when filled with an internal solution containing 140 mM K-gluconate, 10 mM KCl, 1 mM EGTA, 2 mM Na_2-ATP, and 10 mM HEPES, pH 7.3, with KOH. Whole cell patch-clamp recordings were performed in current-clamp mode using a Multiclamp700B Amplifier, digitized with the Digidata 1322A interface and acquired with pClamp 10.2 software (Molecular Devices).

For hM3Dq-expressing GnRH neurons, 1 µM CNO was added in the aCSF bathing medium by the use of the perfusion system after stable baseline recording. Recordings were analyzed using Clampfit 10.2 pClamp software (Molecular Devices). For each recording, the membrane potential and mean firing rate were determined before and during the bath application of drugs. Neurons were considered responsive if there was a > 20% change in firing rate during the course of CNO activation. Only cells which showed less than 20% change in access resistance throughout the recording were included in this study. Junction potential was corrected in the data analysis.

Stereotaxic surgeries for AAV injections

Adult mice were anesthetized with 2% isoflurane and placed in a stereotaxic apparatus (Stoelting Co, Wood Dale, IL) with head and nose fixed. All experiments were performed with sterile instruments and aseptic conditions. Mice were maintained on a warm platform at 37°C during anaesthesia. A small skin incision was performed to expose the animal skull and two injection sites were drilled across the skull to allow injection of AAVs. The skin was then sutured, and animals were allowed to recover for at least two weeks.

Calcium-imaging experiment

In vivo calcium-imaging

Experimental procedures involving animals have been approved by the University of Turin ethical Committee and by the National Council on Animal Care of the Italian Ministry of Health (authorization # 813/2018-PR). All experiments were carried out according to the guidelines of the European Communities Council Directive. All animals were housed under a 12-hour light:dark cycle in individually ventilated cages, with a maximum of 5 animals per cage. Experiments were performed on adult (12–16 weeks old) GnRH::Cre male mice. The adeno-associated viruses (AAVs) pGP-AAV-CAG-FLEX-jGCaMP7s-WPRE (AAV Retrograde, 104405-AAVRg) and pAAV.CAG.Flex.GCaMP6s.WPRE.SV40 (AAV9, 100842-AAV9) were purchased from Addgene. Briefly, mice were anaesthetized by isoflurane inhalation and placed into a stereotaxic apparatus (Stoelting Co, Wood Dale, IL). Mice were maintained on a warm platform at 37 °C during anaesthesia. A small skin incision was performed to expose the animal skull and two injection sites were drilled across the skull to allow injection of AAVs. GnRH positive neurons in the main olfactory bulb were targeted by using the following stereotaxic coordinates: 6.4 mm anterior and 1.2 mm lateral to bregma, Z = 1 mm for the first injection site, and 3.9 mm anterior and 1.8 mm lateral to bregma, Z = 1.5 mm for the second injection site. 500 nl of a mixture pAAV.CAG.Flex.GCaMP6s and pGP-AAV-CAG-FLEX-jGCaMP7 (1:4, 1:2 in saline 0.9% respectively) were slowly injected in each injection site over a period of 30 minutes. The skin was then sutured, and animals were allowed to recover for at least two weeks.

Two-photon functional imaging and odor delivery

Responses of GnRH-positive neurons to odors were recorded and characterized by performing *in vivo* two-photon functional imaging on GCaMP injected mice under anaesthesia. Animals were deeply anesthetized by Ketamine/Xylazine (50 mg/kg, 4mg/kg intraperitoneal administration). The skin was then removed to expose the skull and a metal head-plate was attached to the animal skull and centered over the main olfactory bulb. A small craniotomy (~ 300×300µm^2) was opened on top of the most lateral-posterior part of the main olfactory bulb carefully maintaining the dura intact. The surface of the brain was kept moist with normal HEPES-buffered artificial cerebrospinal fluid. Body temperature was maintained at 37 °C with a heating pad. Depth of anesthesia was assured by monitoring respiration rate, eyelid reflex,

vibrissae movements, and reactions to pinching the tail and toe. All incisions were infiltrated with lidocaine.

Animals were placed under a standard laser scanning two-photon microscope (Nikon A1RMP) couple to a Chameleon Ultra II (Coherent Santa Clara, CA, λexc = 920nm). The activity of GnRH positive neurons was monitored by imaging GCaMP fluorescence, collected via a 16x objective (Nikon CFI75 LWD 16xW NIR A.N.0,80 d.l. 3,0 mm). Signals were acquired by collecting temporal series (t-series) of images at an acquisition frame rate of 1.1-1.5 Hz at laser power ranging between 20 and 40 mW. To test for odor responses in GnRH positive neurons, a custom made olfactometer was used for odors delivery. The system was equipped with a common source of fresh air which was split into two parallel tubing system to either deliver air only or a single odor. The air flow was kept constant throughout the entire recording and odors were delivered by quickly switching between the two parallel ways. The tubing system reached the animal nose at about 0.5 cm. 50 µl of fresh urine from female mice in estrus phase were loaded in the olfactometer and used for cell response characterization. For each recorded cell, at least two control (no odor) and two odor trials of 5 minutes each were run. The odor trial consists of 120 seconds of fresh air, 30 seconds of odor exposure and 150 seconds of fresh air. At the end of each experiment, animals were perfused, and the brains collected for histological analysis.

Two-photon data analysis

Temporal series recorded by two-photon imaging were first imported into the open-source ImageJ/Fiji software (Schindelin et al., 2012) and pre-processed in order to extract the fluorescence signal. A region of interest (ROI) was drawn following the cell profile and the average fluorescence intensity in the defined region was evaluated for each frame. The temporal profile of cell fluorescence was extracted for all trials from each recorded cell and analysed using Matlab software (TheMathWorks). Fluorescent signals were first down-sampled at 1Hz to allow comparison between different neurons acquired at different acquisition frequency and then computed as $dF/F_0=(F_{(t)}-F_0)/F_0$, where $F_{(t)}$ is the fluorescence value at time t, and F_0 is the fluorescence baseline which was calculated as the average intensity across the whole t-series. Only cells with at least three odor trials were considered to evaluate odor responses. For each neuron, traces were aligned with the stimulus onset and averaged. The maximum or the minimum dF/F_0 within 10 seconds after the stimulus onset

was calculated on the averaged trace. Signals exceeding the threshold of baseline-fluorescence ± 3 standard deviations (calculated in the 20 seconds before the stimulus onset) were classified as positively or negatively modulated respectively. Otherwise, the neuron was classified as non-responsive. To test if odors evoked a significant response compared to fresh air presentation, statistical analysis was performed comparing responses to odors and to fresh air only, using the non-parametric paired Wilcoxon signed rank test. Values are expressed as mean ± s.e.m; **p<0.01; n.s. not significant. To evaluate response latency for positively modulated cells, we fitted the calcium trace with a straight line considering the stimulus onset and the peak of response. The intersection between the straight line and the line at zero value of dF/F_0 was considered as response latency.

***In vivo* chemogenetic manipulation**

Stereotaxic coordinates according to the Paxinos mouse brain atlas were + 3.4 mm anterio-posterior, ± 0.3 mm medial-lateral and - 2.6 mm dorsal-ventral to target the olfactory GnRH neurons (glomerular and olfactory nerve layer). Syringes were left *in situ* for 5 min prior to and 10 min following injection. For chemogenetic activation and inhibition studies, mice received simultaneous bilateral injection of 250 nL (50nL/min rate) of a solution mix into the olfactory bulbs containing both AAV9-hSyn-DIO-hM3D(Gq)-mCherry (Addgene #44361-AAV9) (activator DREADD) and KORD (k-opioid receptor D138N mutant; inhibitor DREADD; AAV8-hSyn-dF-HA-KORD-IRES-mCitrine; Addgene #65417-AAV8). pAAV-hSyn-dF-HA-KORD-IRES-mCitrine was a gift from Bryan Roth (Addgene plasmid # 65417; http://n2t.net/addgene:65417; RRID:Addgene_65417).

Stereotaxic coordinates according to the Paxinos mouse brain atlas were -1.9 mm anterio-posterior; ± 2.0 mm medial-lateral and -5.0 mm dorsal-ventral to target the olfactory GnRH neurons sending projections to the medial amygdala. Syringes were left *in situ* for 5 min prior to and 10 min following injection. For chemogenetic inhibition studies, mice received simultaneous bilateral injection of 300 nL (50nL/min rate) of AAV-hSyn-DIO-hM4D(Gi)-mCherry (Addgene #44362). pAAV-hSyn-DIO-hM4D(Gi)-mCherry (Addgene plasmid # 44362) was a gift from Bryan Roth. Bilateral injections were performed using a 2 µL Hamilton syringe (#7000 series; Hamilton Company©, Reno, Nevada, USA).

Cell specific genetic ablation

For Caspase-3 studies, mice received simultaneous bilateral injection of 500 nL (50nL/min rate) of AAV9-flex-taCasp3-TEVp. pAAV-kex-taCasp3-TEVp was a gift from Nirao Shah & Jim Wells (Addgene plasmid # 45580; http://n2t.net/addgene:45580; RRID:Addgene_45580). Viral preparations (AAV9 packaging) were performed by Dr. Emilia Turco at the University of Turin, Italy.

LH ELISA

Following behavior experiments, serial blood samples were collected at the baseline -6 minutes considering time -1 minute as the start of either saline (vehicle solution), Clozapine-N-Oxide (CNO) (1mg/kg) injections or female smell exposure. Briefly, 4 µL of full blood were collected from the tail every 6 minutes over one hour. Samples were collected in 50 µL of 0.01M Phosphate-buffered saline - 0.05% Tween (PBS-T) and immediately snap frozen in dry ice. Blood samples were stored at -80°C until being processed for the LH ELISA (described below). Regarding the testosterone ELISA, blood samples were collected 1h30 either after saline (vehicle solution), CNO (1mg/kg) injections or female smell exposure. Blood was collected from the cheek. Then, samples were centrifuge for 15 minutes at 6.6 rpm at 4°C. The supernatant is pipetted and stored at -80°C until being processed for the testosterone ELISA (described below). Male mice were sexually experienced and trained at least thrice before blood collection.

An ultrasensitive and in-house ELISA was used to measure circulating LH levels as previously reported (Steyn et al., 2013). Firstly, 96-well high-affinity binding microplates (9018; Corning) were coated with a bovine LHβ518B7 monoclonal antibody (1:1000 in 0.01M PBS) provided by Lillian E. Sibley (UC Davis). Circulating hormone levels were determined using a mouse LH-RP reference provided by Albert F. Parlow (National Hormone & Pituitary Program, Torrance, CA, USA). A rabbit polyclonal primary antibody for LH (1:10.000; rabbit antiserum AFP240580Rb; National Hormone and Peptide Program, USA) and a polyclonal goat anti-rabbit IgG secondary antibody (1:1000; DAKO) were also used for this assay. Absorbance was read at a wavelength of 490 nm (Multiskan Ascent Thermo Labsystems, Ascent Software). The concentration of LH in serum samples was determined by interpolating the OD values of unknows against a nonlinear regression of the LH standard curve. The sensitivity of this routine

LH ELISA assay was 0.04 ng/mL. For chemogenetic studies, the average intra-assay coefficient of variation was 3.6% and the inter-assay coefficient of variation was 9.7%.

Testosterone ELISA

A commercially available mouse testosterone ELISA kit was used to measure plasma testosterone levels (DEV9911; Demeditec Diagnostics, GmnH) in accordance with manufacturer's instructions. The ELISA sensitivity was 0.066 ng/mL and the intra-assay coefficient of variation for testosterone was 9.09%.

Focal exposure with female bedding

The experiment was carried out in the Laboratory of Dr. Ulrich Boehm (University of Saarland School of Medicine, D-66421 Homburg, Germany). Two groups of GnRHR::IRES-Cre (GRIC)/eR26-τGFP male mice were used, each composed of 4 animals: one group was exposed to the female-soiled bedding and urine for 30 minutes and sacrificed 90 minutes later (to detect cFos activation); the other group was exposed to water for 30 minutes and sacrificed 90 minutes later (controls). The female-soiled bedding used for the stimulus was composed of a mixture of soiled bedding and urine, collected from the cages of C57BL6/J estrous females.

Pheromonal stimulation

We tested the ability of urinary pheromones to activate GnRH expressing neurons located in different areas of the brain. Urine presented to stimulate the animals and for the olfactory preference test was collected from sexually experienced males and from estrous females. Male and female (in estrus) urine was collected and stored at -80°C until the day of use. Soiled bedding from sexually experienced males and estrous females were also collected for pheromonal stimulation. Soiled bedding was then stored in a closed bag at -20°C.

Behavioral Assessment

Olfactory preference test

For all behavioral tests, the animal subjects were coded, the investigator was blind to the genotype of each animal. During the social olfactory preference test assessment male mice were first exposed to urine from an adult C57BL/6J WT stud male and an estrus female for a

habituation period of 30 min. After a dishabituation period: 30 min in clean bedding, the same urine samples were presented to the mice. Urine was delivered on a piece of filter paper placed within a Petri dish (enabling direct contact with the source). Male and female urine samples were placed on opposite sides of the cage, equidistant from the cage walls. Trials lasted 10 min, during which mouse behavior directed towards the two urine sources was recorded. The amount of time spent sniffing the Petri dish was used as an indication of the mouse's interest gaining further information from a scent source. The tests were conducted in the animals' home cages to minimize both manipulation and exposure to external stimuli. For each test, 50 µl of either male or female urine were put on each piece of filter paper. Each session was video-recorded, and the time the animals spent sniffing the urine source was subsequently measured by BORIS software.

Habituation/dishabituation test

Odor habituation/dishabituation was used to measure non-social olfactory discrimination (De Chevigny et al., 2006; Breton-Provencher, 2009). Briefly, during the dark phase, mice were familiarized with a first odor (habituation odor) during four successive sessions and then exposed once to a novel odor (dishabituation odor). Each session was 1 minute long and was followed by a 10 minutes inter-session interval. Olfactory discrimination was analyzed using two different odors: acetophenone (00790, Sigma) and octanal (05608, sigma), which were diluted 10^{-3} % in mineral oil (M3516, Sigma) and delivered via patches of filter paper. Twenty microliters of odor were applied to each filter paper, which was then placed in a petri dish. The two odors were both alternatively tested as novel stimuli (during dishabituation) in separate sessions. Mouse sniffing activity was quantified using BORIS software (Friard and Gamba, 2016).

Olfactory detection threshold

Olfactory detection threshold test was used to determine the lowest concentration of female urine that is perceivable by the sense of smell. This test was performed during the dark phase. During each session mice are placed in clean cages, and placed back in their own cage during the inter-session interval. Each session was 1 minute long and was followed by a 10 minutes inter-session interval. Olfactory detection threshold was analyzed using increased concentrations of the female urine scent (diluted in saline at 1:1000; 1:100: 1:10; pure), to

determine the minimum concentration that triggers exploration by the animal. Mouse sniffing activity was quantified using BORIS software (Friard and Gamba, 2016).

Open field test and odor exposure

The open field test was used to assess olfactory preference and aversive behavior. Mice were placed in the center of the apparatus (45 x 45 x 34 cm^3) and allowed to explore freely the arena for 10 minutes during the habituation period. After 10 minutes, an odor was placed in the center of the area (saline, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), pure female urine. Female urine has been collected one to two week(s) before the test during the estrus phase, and stored at -20°C. Test lasted 10 minutes. Mouse sniffing activity was quantified using BORIS software (Friard and Gamba, 2016) and mouse's tracking activity was analyzed by a video tracking system (Ethovision XT 14.0, Noldus, Wageningen, The Netherlands).

Buried odor test

Mice were individually introduced into a clean cage containing 3cm deep of clean bedding with a small piece of filter paper with 50µl of pure estrus female urine buried beneath 1 cm in a random corner of the cage. Time spent to retrieve the filter paper (latency) was measured. If the mouse failed to find the buried smell within 5min, the test was stopped and the latency score was recorded as 300sec. This test was performed during the dark phase of mice's cycle.

Sexual behavior test

Male mice were tested for sexual behavior over the dark phase following previously reported protocols (Liu et al., 2020). All behavior tests were videotaped and manually annotated using BORIS software.

Elevated plus maze (EPM) test

The Elevated Plus Maze (EPM) was used to assess the anxiety behavior of the mice. The equipment consisted of two open arms and two enclosed arms by 14.5-cm-high walls forming an elevated cross-positioned 50 cm above the floor (36.5 × 6 cm). The mouse was placed in the middle of the maze and allowed to explore the maze for 10 min. A video tracking system (Ethovision XT 7.0, Noldus, Wageningen, Netherlands) recorded movements to calculate the percentage of time spent in the open arms, used as an index of anxiety behavior.

Spontaneous locomotor activity

Spontaneous locomotor activity was assessed in an open field test, using an infrared actimeter (Bioseb, Vitrolles, France). The apparatus consisted of a square arena (45 × 45 cm) with a black polymethyl methacrylate floor and transparent 34-cm-high polymethyl methacrylate walls. Mice were placed in the center of the arena and allowed to explore freely for 10 min. Activity was recorded by two rows of infrared photocell sensors and processed with the Actitrack software (Bioseb).

Statistical analysis

All analyses were performed using Prism 9 (GraphPad GraphPad; San Diego, CA, USA) and assessed for normality (Shapiro-Wilk test) and variance, when appropriate. Statistically significant *P* values were considered when $P < 0.05$. Sample sizes were chosen according to standard practice in the field. For normal distribution, data were compared using unpaired two-tailed Student's t-test and one-way and two-way ANOVA followed by a Sidak's *post hoc* test. For non-normally distributed values, Mann–Whitney and Kruskal–Wallis (one-way analysis) tests were used. Wilcoxon signed-rank test was used in all paired non-parametric analysis comparing before and after drug treatment during olfactory preference tests.

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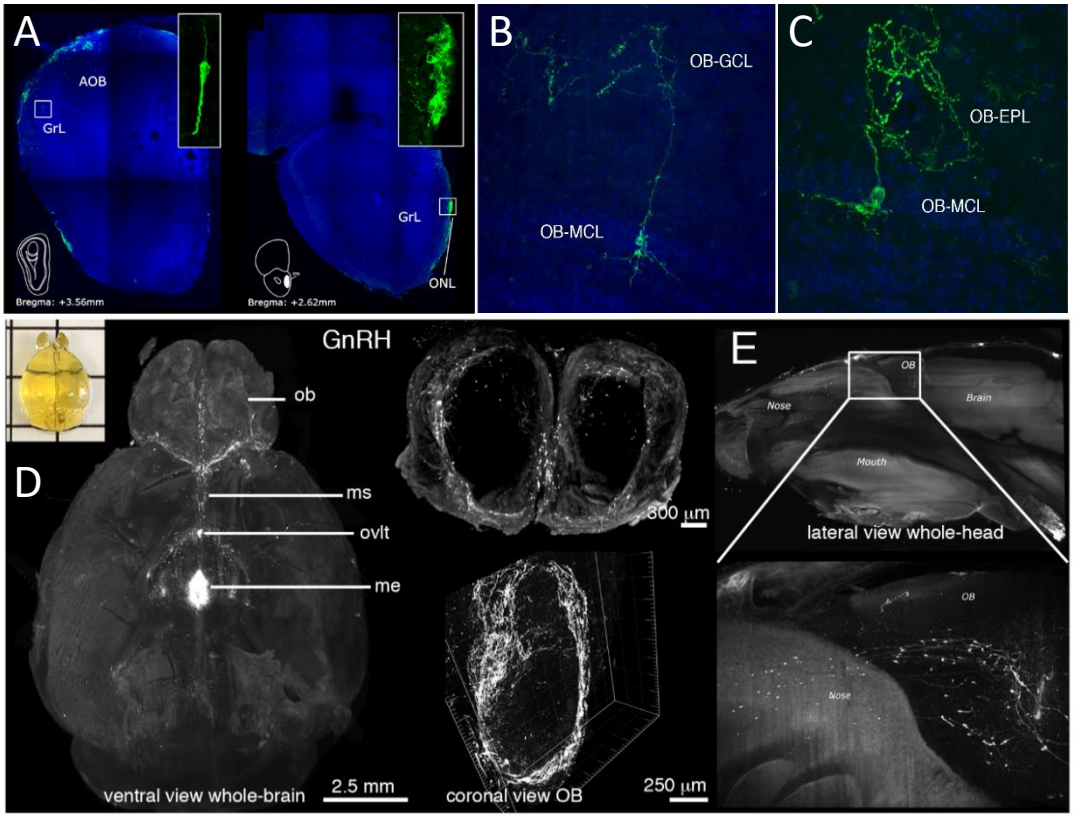
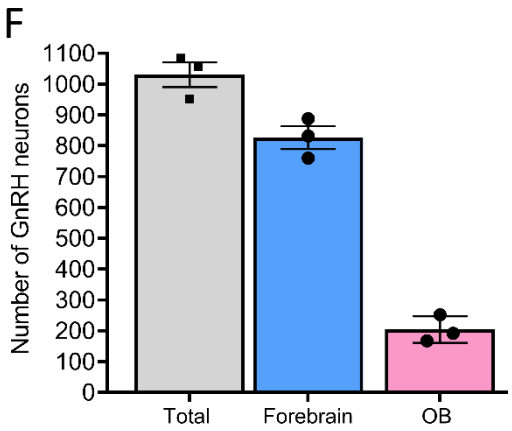
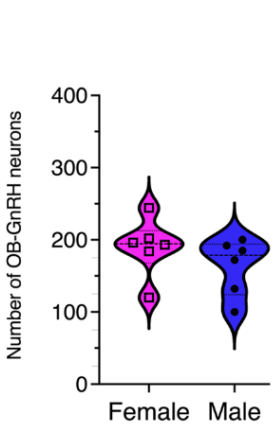
Chapter 5: Results

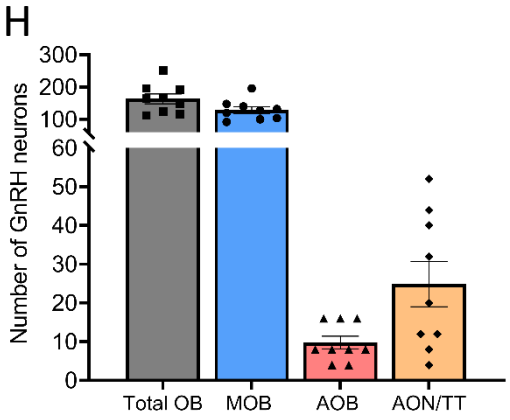
GnRH neurons constitute a diverse population in the olfactory bulbs.

During fetal development, GnRH neurons migrate from the medial nasal placode to several brain regions including the preoptic area and the olfactory bulbs (Casoni et al., 2016). We first confirmed the presence of the GnRH neuronal population within the olfactory bulbs using an antibody directed against the GnRH peptide (**Figure 1A**). To further examine the distribution of OB GnRH neurons, we used immunofluorescence labeling with the GnRH antibody on floating sections and whole-mount immunolabeling using iDISCO+ technology (**Figure 1**). We found that the GnRH neuronal population in mouse is scattered through the olfactory bulbs and consisted of about 200 neurons in both males and females ($female_{(N=6)}$ = 189.8 ± 40.1 vs $male_{(N=6)}$ = 176.7 ± 40.25 neurons; P = 0.277; **Figure 1 F, G**), representing nearly 20% of the entire GnRH population in the mouse brain. iDISCO+ immunolabeling confirmed the presence of GnRH neurons and fibers in the caudal region of the olfactory bulbs, these results are in accordance to what was observed previously in the lab, by Casoni et al., 2016. The majority of OB GnRH neurons are located in the MOB, with smaller populations within the AOB, the accessory olfactory nucleus (AON) and the ventral part of the tenia tecta (TT), two components of the olfactory cortex (**Figure 1H**). Within the MOB, OB GnRH neurons are mainly located within the olfactory nerve layer (ONL), the glomerular cell layer (GL), the granular cell layer (GrL), and few neurons are found within the mitral cell layer (MCL) and the external plexiform cell layer (EPL). Depending on their location, the OB GnRH neurons displayed heterogenous morphologies. The majority exhibit the classic bipolar shape of GnRH neurons in the GL, the GrL, the AOB, they are round and cluster in the ONL (**Figure 1A**), and multipolar in proximity to the MCL from which dendritic trees extended into the EPC (**Figure 1B, C**).


A
AOB
GrL
Bregma: +3.56mm
GrL
ONL
Bregma: +2.62mm
B
OB-GCL
OB-MCL
C
OB-EPL
OB-MCL
D
GnRH
ob
ms
ovlt
me
ventral view whole-brain
2.5 mm
300 µm
coronal view OB
250 µm
E
OB
Nose
Brain
Mouth
lateral view whole-head
OB
Nose


F
Number of GnRH neurons
1100
1000
900
800
700
600
500
400
300
200
100
0
Total
Forebrain
OB


G
Number of OB-GnRH neurons
400
300
200
100
0
Female
Male


H
Number of GnRH neurons
300
200
100
60
50
40
30
20
10
0
Total OB
MOB
AOB
AON/TT

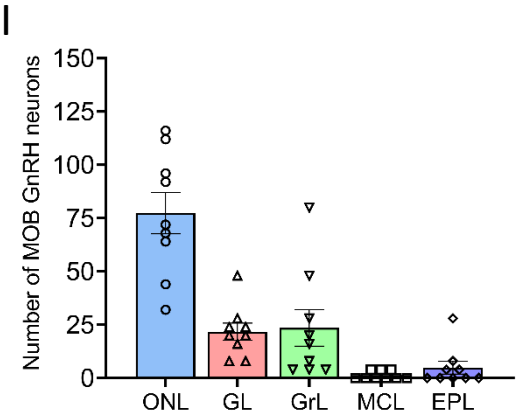
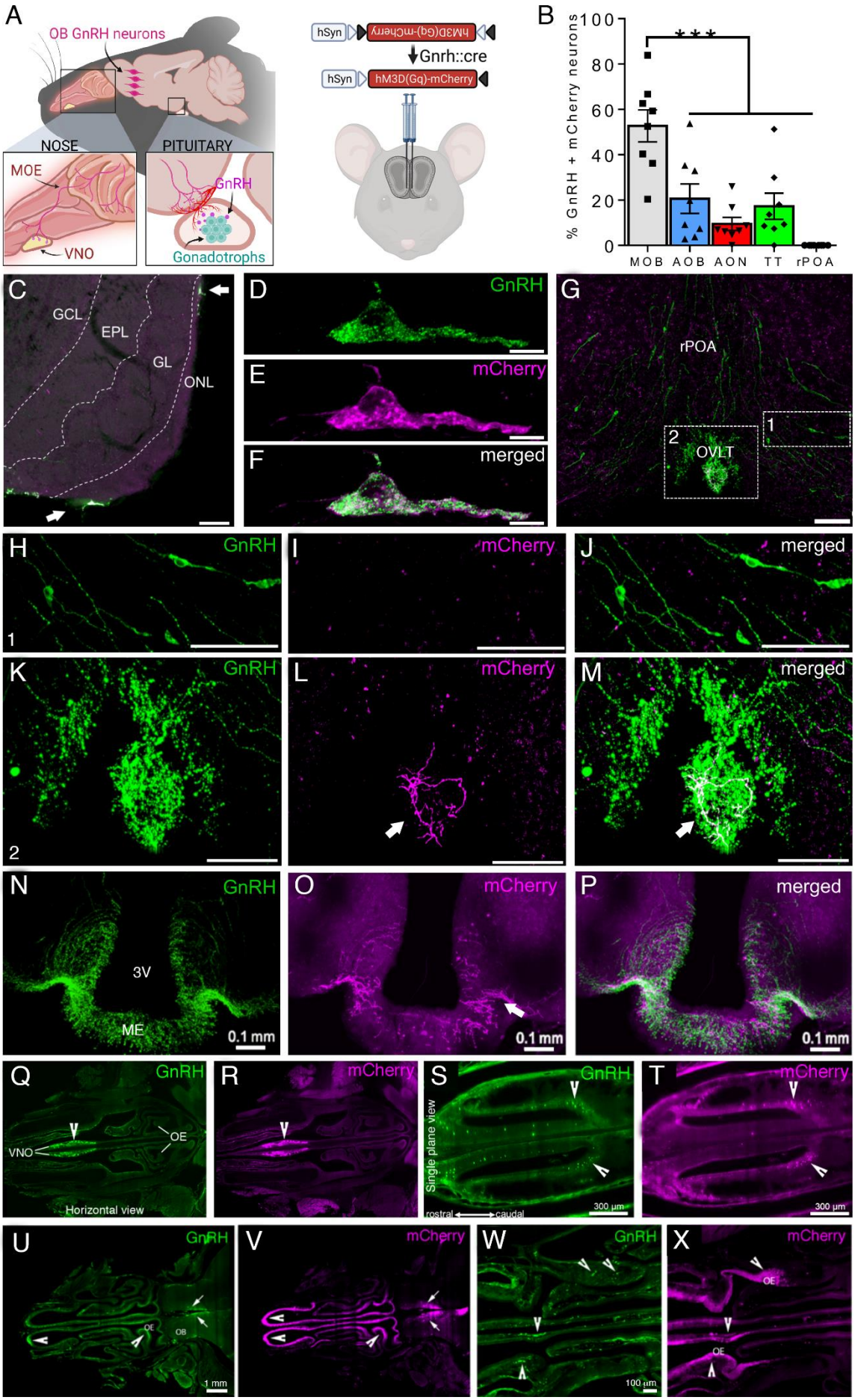

I
Number of MOB GnRH neurons
150
125
100
75
50
25
0
ONL
GL
GrL
MCL
EPL

Figure 1: Distribution and number of GnRH neurons within the olfactory bulbs. **(A-C)** Representative confocal images (coronal sections) illustrating different morphologies of OB GnRH neurons. **(D)** iDISCO+ and 3D-imaging of an entire mouse brain (male) showing immunoreactive GnRH neurons and fibers in the OB (ventral and coronal views), along the MS, OVLT, and reaching the ME; N=3; 12-16 weeks-old males. **(E) Upper part:** Whole head lateral view of the nose and the brain of a male mouse. **Lower part:** zoom in the OB showing the presence of GnRH neurons and fibers within the olfactory areas. **(F)** Quantitative analysis representing the total number of GnRH neurons in the brain, forebrain and OB. (N=3). **(G)** Quantitative analysis representing the mean number of GnRH neurons distributed in the OB of male and female mice (N=6; Student t-test; $P > 0.5$). **(H)** Quantitative analysis of OB GnRH neuronal distribution in the MOS and the AOS of adult male mice (N=9; 16-20 weeks-old males). **(I)** Quantitative analysis of OB GnRH neurons throughout different layers of the MOB (N=9). Values are indicated as mean ± SEM. ** $P < 0.01$; **** $P < 0.0001$. Scale bars: **(A)** 100 µm, inset 10 µm; **(B)** 30 µm; **(C)** 20 µm; **(D)** left figure 2.5 mm, right upper figure 800 µm, right lower figure 250 µm. OB: olfactory bulbs; MS: medial septum; OVLT: organum vasculosum of the lamina terminalis; ME: median eminence; AOB; accessory olfactory bulbs; GrL: granule cell layer; ONL: olfactory nerve layer; AON: accessory olfactory nucleus; TT: tenia tecta; GL: glomerular cell layer; MCL: mitral cell layer; EPL: external plexiform layer.

OB GnRH neurons projections to hypothalamic and olfactory areas

We next mapped the OB GnRH neuronal projections by specifically infected the OB GnRH neurons with a Cre-dependent adeno-associated virus (AAV) expressing mCherry under the control of human synapsin (hSyn) promoter performing bilateral stereotaxic injections in the olfactory bulbs (**Figure 2A**). This approach yielded an average of 52.72 ± 7.04% of OB GnRH neurons co-expressing GnRH and mCherry within the MOB, and about 20% of GnRH/mCherry co-expression in each of the other areas (AOB, AON, TT) ($P < 0.001$; **Figure 2B-F**). We did not detect any viral expression in GnRH neurons located in the rostral preoptic area (rPOA), although OB GnRH neurons extend projections to the organum vasculosum laminae terminalis (OVLT; bregma = +0.5 mm; **Figure 2G-M**). Most OB GnRH neurons send projections innervating the ME, suggesting that these cells may be hypophysiotropic (**Figure 2N-P**). Since OB GnRH neurons are often bipolar-shaped, with two neurites extending in opposite directions, we then examined the possibility that one of such dendrites could extend into the nasal region and eventually terminate within the olfactory structures. We took advantage of iDISCO+ immunostaining of whole decalcified heads to address this question. This strategy confirmed that OB GnRH neurons send neurites into the VNO (arrowheads) (**Figure 2Q-T**) and the main olfactory epithelium, MOE (arrowheads) (**Figure U-X**), indicating anatomical connectivity between the OB GnRH neurons and these olfactory/pheromonal structures.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% GnRH + mCherry neurons
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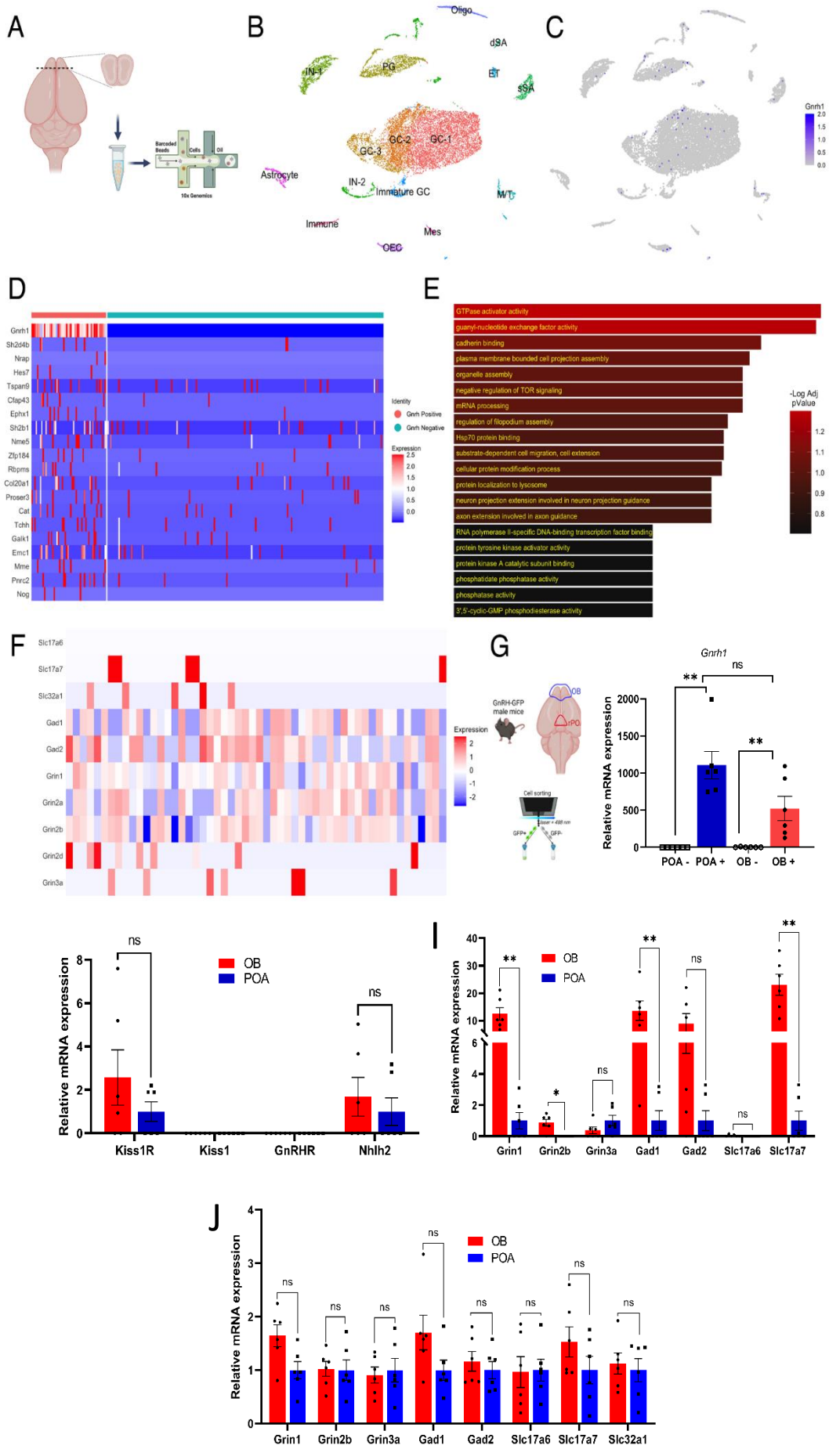
Figure 2: OB GnRH neurons connect the olfactory/vomeronasal structures with the hypothalamus. **(A)** Schematic of the experimental strategy for selective expression of AAV9-mCherry targeted to OB GnRH neurons in *Gnrh1*$^{cre/wt}$ male mice. **(B)** Quantification of OB GnRH neurons infected by the viral tracer throughout the OB and the rPOA. N = 8, age: P90; *** $P < 0.001$; two-way ANOVA; Tuckey's multiple comparisons test; values indicate means ± SEM. **(C)** Representative coronal section of the OB representing GnRH-immunoreactive (green) and mCherry-positive cells (magenta) within the ONL. **(D-F)** High magnification of ONL GnRH cell showing GnRH, mCherry and merged respectively. **(G)** Representative coronal section shows the expression of GnRH (green) and mCherry (magenta) in the rPOA. **(H-M)** High-power micrographs of white boxes 1 **(H-J)** and 2 **(K-M)** in G. **(N-P)** GnRH and mCherry expression in the ME. **(Q-X)** iDISCO+ immunostaining for GnRH and mCherry of whole decalcified heads (N = 3 males, P90). **(Q-T)** Representative optical transversal planes of the nasal region showing the expression of GnRH and mCherry in the VNO (arrowheads). **(U to X)** Representative optical transversal planes of the nasal region showing the expression of GnRH and mCherry in the OE (arrowheads) and in the OB (arrows). Scale bars: **(C)**, **(D-F)**, **(G)**, **(H-J)**, **(K-M)**, **(N-P)** 100 μm, **(Q-R), (S-T)** 300 μm, **(U-V)** 1 mm, **(W-X)** 100 μm. OB: olfactory bulbs, MOE: main olfactory epithelium; VNO: vomeronasal organ; AOB: accessory olfactory bulbs, AON: accessory olfactory nucleus, TT: tenia tecta, rPOA: rostral preoptic area, GCL: granular cell layer, EPL: external plexiform layer, GL: glomerular cell layer, ONL: olfactory nerve layer, OVLT: organum vasculosum laminae terminalis, 3V: third ventricle, ME: median eminence, OE: olfactory epithelium.

Comparison of OB and POA GnRH neurons transcriptomic profiles

To characterize the cellular and molecular features of the OB GnRH neurons in male mice, we performed single-nucleus RNA sequencing (snRNAseq) to 13,163 cells collected from the OB of adult male mice (18 weeks-old; **Figure 3A**). Cells were clustered into 16 cell types and annotated them using the expression levels of cluster-enriched genes, and assigned identities to each one of them as canonical marker expression (Nagayama et al., 2014). We detected the presence of the major OB cell populations, including periglomerular cells (PG), granule cells (GC), mitral/tufted cells (M/T), inhibitory neurons (IN), superficial Short Axon Cells (sSA), deep Short Axon Cells (dSA), astrocytes, olfactory ensheathing cells (OEC) (**Figure 3B**). Within the data set, 54 *Gnrh1* positive cells were identified, corresponding to roughly 0.4% of the total OB cell population. Expression data from these neurons were projected into two-dimensional

space using Uniform Manifold Approximation and Projection (UMAP) algorithms, which visualizes clustered cells with similar transcriptional programs (**Figure 3C**). We next generated heatmaps showing the expression patterns of the top 20 up-regulated genes in *Gnrh1* positive cells as compared to *Gnrh1* negative cells (**Figure 3D**). By Gene Ontology (GO) analysis, we identified enrichment in *GnRH1* neurons in genes involved in cytoskeletal rearrangement, intracellular signaling, axon guidance and neuron projections (**Figure 3E**).

We next have a look at the neurotransmitter expression profile of OB GnRH neurons. Results revealed that OB GnRH neurons express glutamatergic markers such as *Grin1*, encoding for the glutamate ionotropic receptor NMDA type 1 subunit; *Scl17a7,* encoding for the vesicular glutamate transporter type 1, vGUT1; but not *Scl17a6* encoding for vGLUT2. In addition, OB GnRH neurons express GABAergic markers such as *Gad1* (glutamate decarboxylase 1) and *Gad2* (glutamate decarboxylase 2) (**Figure 3F**). Then, we used fluorescent activated cell sorting (FACS) to isolate GnRH cells from the olfactory bulbs and the preoptic area of GnRH-green fluorescent protein (GFP) male mice (Spergel et al., 1999) and performed RT-qPCR analysis to validate the single-nucleus RNA sequencing data on OB GnRH neurons and to compare the neurotransmitter expression profiles between OB and POA GnRH neurons. As expected, we found high enrichment of *Gnrh* transcript in GFP+ cells isolated from the OB and POA as compared with the GFP- cells (**Figure 3G**). To validate the sorting and the RT-qPCR analysis we aimed to confirm the expression of genes known to be expressed, or not, in GnRH neurons such as *Kiss1r*, *Nhlh2*, *Kiss1* and *Gnrhr*. Our results confirmed that GnRH neurons from both populations express *Kiss1r* and *Nhlh2*, whereas they do not express *Kiss1* and *Gnrhr* (**Figure 3H**). Next, we aimed to confirm and compare the neurotransmitter expression between OB and POA GnRH neurons. We found a significantly higher expression of glutamatergic (*Grin1*, *Grin2b* and *Slc17a7*) and GABAergic (*Gad1* and *Gad2*) transcripts in OB GnRH as compared to POA GnRH neurons but higher expression of the glutamate ionotropic receptor NMDA type 3a subunit (*Grin3a*) in POA GnRH neurons as compared to OB GnRH neurons (**Figure 3I**).
Next, we evaluated whether sexual experienced modify the neurotransmitter transcriptomic expression profiles of OB and POA GnRH neurons. Results demonstrated that sexual experience abolished all the differences observed in gene expression profiles between OB and POA GnRH neurons (**Figure 3J**).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Proser3
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Tchh
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Mme
Prrc2
Nog
Identity
Gnrh Positive
Gnrh Negative
Expression
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GTPase activator activity
guanyl-nucleotide exchange factor activity
cadherin binding
plasma membrane bounded cell projection assembly
organelle assembly
negative regulation of TOR signaling
mRNA processing
regulation of filopodium assembly
Hsp70 protein binding
substrate-dependent cell migration, cell extension
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protein localization to lysosome
neuron projection extension involved in neuron projection guidance
axon extension involved in axon guidance
RNA polymerase II-specific DNA-binding transcription factor binding
protein tyrosine kinase activator activity
protein kinase A catalytic subunit binding
phosphatidate phosphatase activity
phosphatase activity
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Slc17a7
Slc32a1
Gad1
Gad2
Grin1
Grin2a
Grin2b
Grin2d
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Expression
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GnRH-GFP male mice
OB
rPO
Cell sorting
Gnrh1
Relative mRNA expression
POA -
POA +
OB -
OB +
Kiss1R
Kiss1
GnRHR
Nhlh2
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Grin1
Grin2b
Grin3a
Gad1
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Slc17a6
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Figure 3: Transcriptome profiling of GnRH neurons. (A) Schematic representing single-cell RNA sequencing protocol. **(B)** UMAP visualization of the OB; colored by major cell types. **(C)** UMAP visualization of *Gnrh1* expressing cells in the OB (blue dots; other cell types in grey). **(D)** Transcriptomic landscape of GnRH positive cells compared with GnRH negative cells in the OB. **(E)** Top Go terms of genes with significantly increased differential expression in OB Gnrh cells (Wilcoxon Ranked Sum Test). **(F)** Relative transcriptional expression level of 10 genes involved in glutamatergic or GABAergic signaling in 54 OB GnRH neurons. **(G)** Schematic strategy for sorting OB and POA GnRH-GFP cells from *Gnrh::Gfp* sexually naïve male mice. Relative expression of *Gnrh1* in OB and POA positive and negative sorted cells. **(H)** Relative mRNA expression of *Kiss1r*, *Kiss1*, *Gnrhr* and *Nhlh2* in OB^{GFP+} and POA^{GFP+} sorted cells. Gene expression levels were normalized on POA levels. **(I)** Comparison of mRNA expression level of *Grin1, Grin2b, Grin3a, Slc17a6, Slc17a7; Gad1, Gad2* in *Gnrh* positive cells isolated from the OB and the POA. **(J)** Comparison of mRNA expression level of *Grin1, Grin2b, Grin3a, Slc17a6, Slc17a7; Gad1, Gad2*; *Scl32a1* in *Gnrh-gfp* positive cells isolated from the OB and the POA of sexually experienced male mice. Values are represented as the mean ± SEM. N = 6; 12 weeks-old males; ** $P < 0.01$; non-parametric paired Wilcoxon signed rank test. FACS: Fluorescence activated cell sorting; OB: olfactory bulbs; rPOA: rostral preoptic area; GFP: green fluorescent protein.

Since OB GnRH neurons extend neurites inside the VNO and the MOE, we assessed whether these neurons can directly detect olfactory cues by expressing vomeronasal and/or olfactory receptors. First, we performed RT-qPCR analysis on *Gnrh::gfp* sorted cells from the OB and the POA to evaluate whether OB GnRH neurons are expressing specific olfactory and vomeronasal receptors known to be essential to mediate olfactory signal transduction in response to same-sex or opposite-sex odors. Results showed that neither OB nor POA GnRH neurons express any of the olfactory and vomeronasal receptors selected. However, the expression of the adenylate cyclase 3 (*Adcy3*), an enzyme involved in olfactory signal transduction and TRPC2, a transduction channel activated through signaling cascades initiated by the interaction of pheromones with V1R and V2R G protein-coupled receptors on the dendrites of the sensory neurons, have been identified in few samples of GnRH positive cells collected from sexually experienced male mice brains (**Figure 4A**).

Secondly, snRNAseq dataset was used as a more general approach to investigate the expression levels of vomeronasal and olfactory receptors (Vmnrs and Olfrs) in *Gnrh1* positive and *Gnrh1* negative cells. The heatmap analysis showed that several Vmnr and Olfr are expressed in the male OB and 14 vomeronasal receptors are found to be expressed by OB GnRH neurons, with a preponderance of the vomeronasal 2 receptor gene family. The genes *Vmn2r1*, *Vmn2r2* and *Vmn2r87* show the highest percentage of expression in *Gnrh* positive cells (**Figure 4B**). Moreover, 26 olfactory receptor genes are expressed in OB GnRH neurons. The Olfr323 is particularly enriched in these neurons (**Figure 4C**).

The expression of the highly enriched transcripts found in OB GnRH neurons, *Vmn2r1* and *Olfr323*, was validated by fluorescent in situ hybridization (FISH) using the RNAscope technology (**Figure D-F**). Our FISH experiments confirmed the expression of *Vmn2r1* in the basal layer of the adult mouse VNO and the expression of the *Olfr323* in the olfactory epithelium (**Figure D-F**). In agreement with our snRNAseq data, we found that some *Gnrh1*-positive neurons located in the OB co-express *Vmn2r1* and *Olfr323*, while majority of GnRH neurons did not express those transcripts (**Figure 4G-J**).

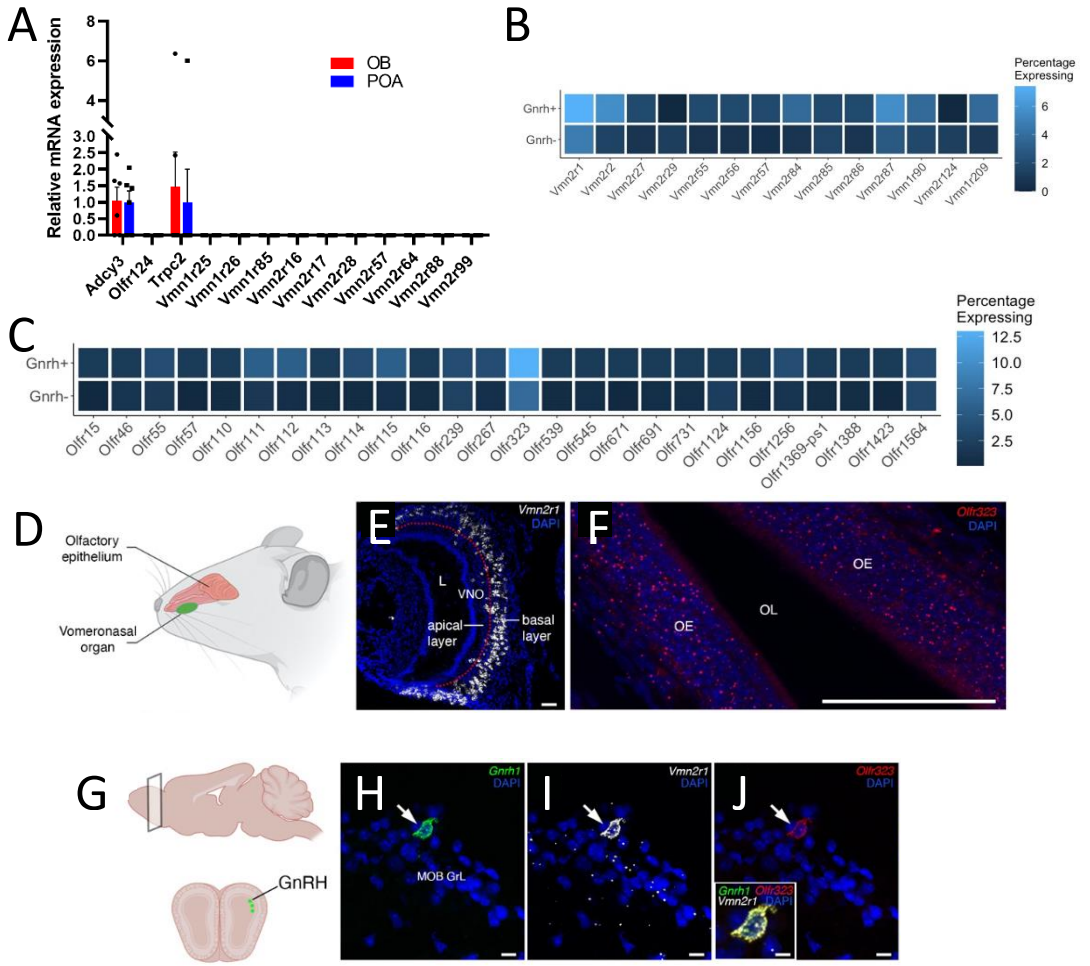


Figure 4: OB GnRH neurons express olfactory and vomeronasal receptors. **(A)** Relative mRNA expression of *Adcy3, Olfr124, Trpc2, Vmn1r25, Vmn1r26, Vmn1r85, Vmn2r16, Vmn2r17, Vmn2r28, Vmn2r57, Vmn2r64, Vmn2r88, Vmn2r99* in OB^{GFP+} and POA^{GFP+} sorted cells. Gene expression levels were normalized on POA levels. **(B)** Percentage expression profile of vomeronasal receptors in OB GnRH positive cells as compared with OB GnRH negative cells. **(C)** Percentage expression profile of olfactory receptors in olfactory GnRH positive cells as compared with olfactory GnRH negative cells. **(D)** Schematic of the murine olfactory epithelium and vomeronasal organ. **(E)** Representative coronal section of a VNO from an adult male mouse (P90) showing expression of *Vmn2r1* mRNA (white staining) by RNAscope. The red dotted line indicates the boundary between the basal and apical layer of the VNO. **(F)** Representative sagittal section of an olfactory epithelium from an adult male mouse (P90) showing expression of Olfr323 mRNA (red) by RNAscope. **(G)** Schematic representation of the murine olfactory bulbs. The GnRH cell depicted on H-J located in the granular cell layer of the olfactory bulb appears in green. **(H-J)** Representative coronal sections of the OB from adult male mice (P90) showing expression of *Gnrh1* (green), *Vmn2r1* (white) and *Olfr323* (red) in the main olfactory bulb granular layer (MOB GrL). N = 3 mice were used for the FISH experiments. Scale bars: **(E)**, 50 µm; **(F)** 100 µm; **(H-J)** 10 µm; **(R)** 5 µm.

Similarly, we assessed whether OB GnRH neurons express hormonal receptors in order to detect the presence of these hormones. snRNAseq dataset confirmed the expression of the androgen receptor (AR) and the progesterone receptor (PR) in OB GnRH neurons, however the estrogen receptors α (ERα) and β (ERβ) are not expressed by OB GnRH neurons according to these data (**Figure 5A**). Again, these results were confirmed by RNAscope (**Figure 5 B-H**). As expected, our data demonstrated that OB GnRH neurons express both the androgen and progesterone receptors mRNA (**Figure 5B-E**) but not ERα mRNA (**Figure 5F-H**). The presence of the androgen and progesterone receptor proteins has to be confirmed by immunofluorescence.

To conclude on this part, these data provide new insights on OB GnRH neurons and suggest that these neurons may respond to olfactory cues to potentially relay olfactory information to central brain areas involved in reproductive physiology and behavior.

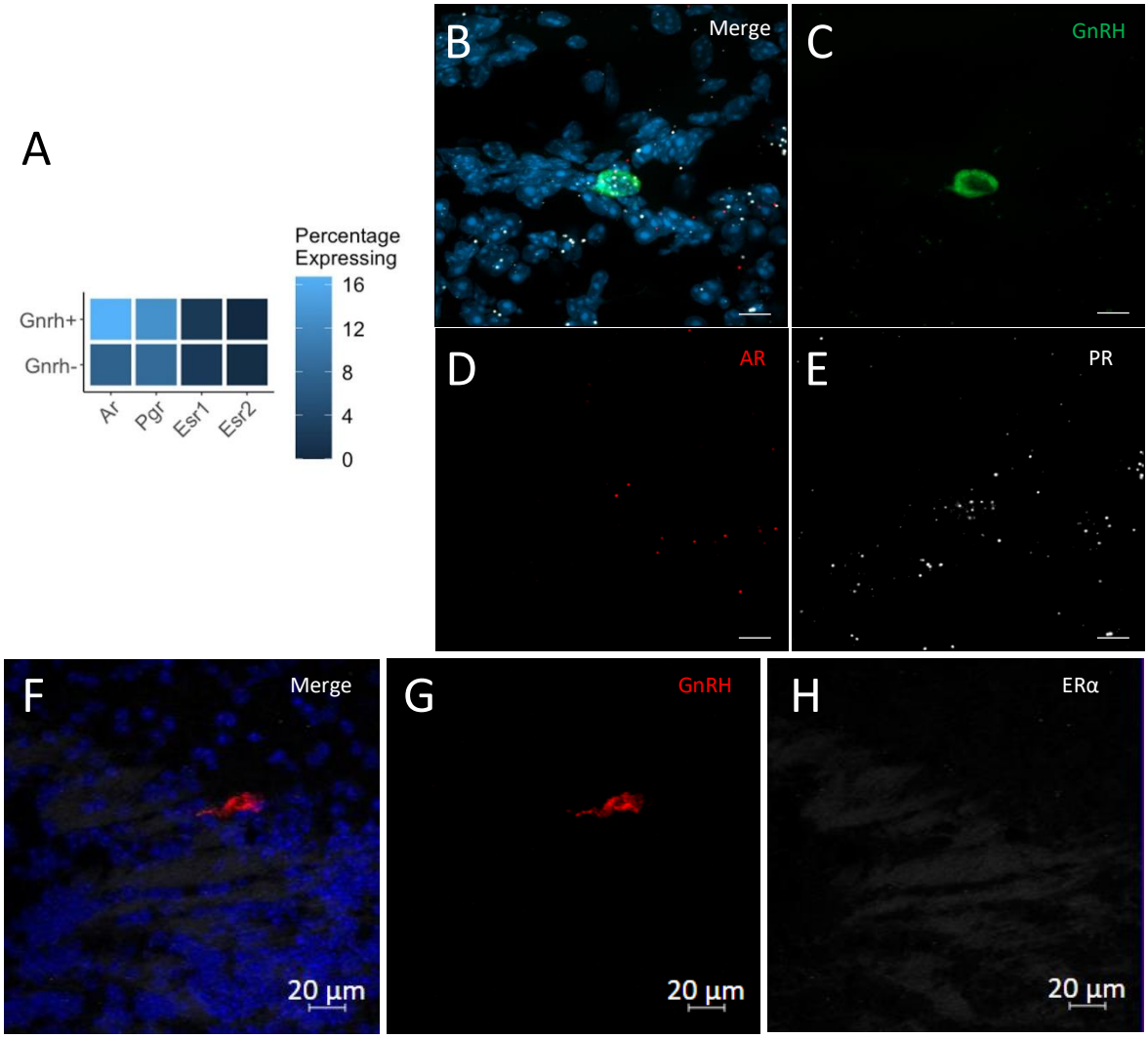


Figure 5: OB GnRH neurons and hormone receptors expression. **(A)** Percentage expression profile of hormone receptors in OB GnRH positive cells as compared with OB GnRH negative cells from single nuclei RNA sequencing. **(B-E)** Representative coronal section of the OB of adult mice (P90) showing mRNA expression of *Gnrh1* (green; **C**), *AR (red;* **D**), *PR* (white; **E**) and merge (**B**). (**F-H**) Representative coronal section of the OB of adult mice (P90) showing expression of *Gnrh1* (red; **G**) and *ERα* (white; **H**) mRNA by fluorescent *in situ* hybridization and **(I-J)** protein expression of GnRH and AR by immuno-histochemistry.

OB GnRH neurons are activated upon the presence of opposite-sex odors *in vivo*

In light with previous findings and to understand what is the specific role of OB GnRH neurons *in vivo*, we next tested whether OB GnRH neurons respond to olfactory cues present in female urine by performing *in vivo* two-photon functional imaging of OB GnRH neurons of sexually experienced males exposed to female estrus urine (**Figure 6A**). Adult *Gnrh1*$^{cre/wt}$ male mice were injected with Cre-dependent AAVs targeting the dorsolateral and posterior part of the OB to express the genetically encoded calcium indicator GCaMP (co-infusion of GCaMP6s and GCaMP7s) selectively in OB GnRH neurons. We first confirmed the specific expression of GCaMP in GnRH-positive neurons by immunohistochemical analysis (**Figure 6B-E**). Two weeks after GCaMP injection, head-fixed mice were placed under the two-photon microscope. OB GnRH neurons were identified by looking at the GCaMP fluorescence and odor responses were characterized during acute exposure to female urine. Female odor-mediated responses were compared to fresh air exposure. We recorded a total of 25 OB GnRH neurons from 8 male mice (**Figure 6F**) and we classified their responses to female urine based on their change in fluorescence within 10s after stimulus onset (Galliano et al., 2018). Among all recorded cells, 36% (9 out of 25 cells) were activated by exposure to female urine showing a significant increase in dF/F_0, compared with exposure to fresh air (dF/F_0 = 0.2094 ± 0.0738 vs 0.7916 ± 0.2528; fresh air vs female urine; N = 9; *P* = 0.0039, Wilcoxon signed-rank test; **Figure 6G**). These enhanced neuronal activities exhibited different patterns, being either transient (**Figure 6G**, right-most trace) or long-lasting (**Figure 6G**, left-most trace) activation, with an average response latency of 1.78s ± 0.786s following the presentation of olfactory stimuli. Of the remaining recordings, 20% of OB GnRH neurons (5 out of 25 cells) showed a tendency toward inhibition in response to female odor (**Figure 6H**; fresh air vs female urine: -0.0914 ± 0.0726 vs -01769 ± 0.1035; N = 5; *P* = 0.0625, Wilcoxon signed-rank test). The last 44% (11 out of 25 cells) did not respond to female urine (**Figure 6I;** fresh air vs female urine: 0.1195 ± 0.0432 vs 0.1155 ± 0.0227; N = 11; *P* = 0.6417, Wilcoxon signed-rank test). These data show that the activity of a sub-population of OB GnRH neurons can be modulated by exposure to opposite-sex urine.

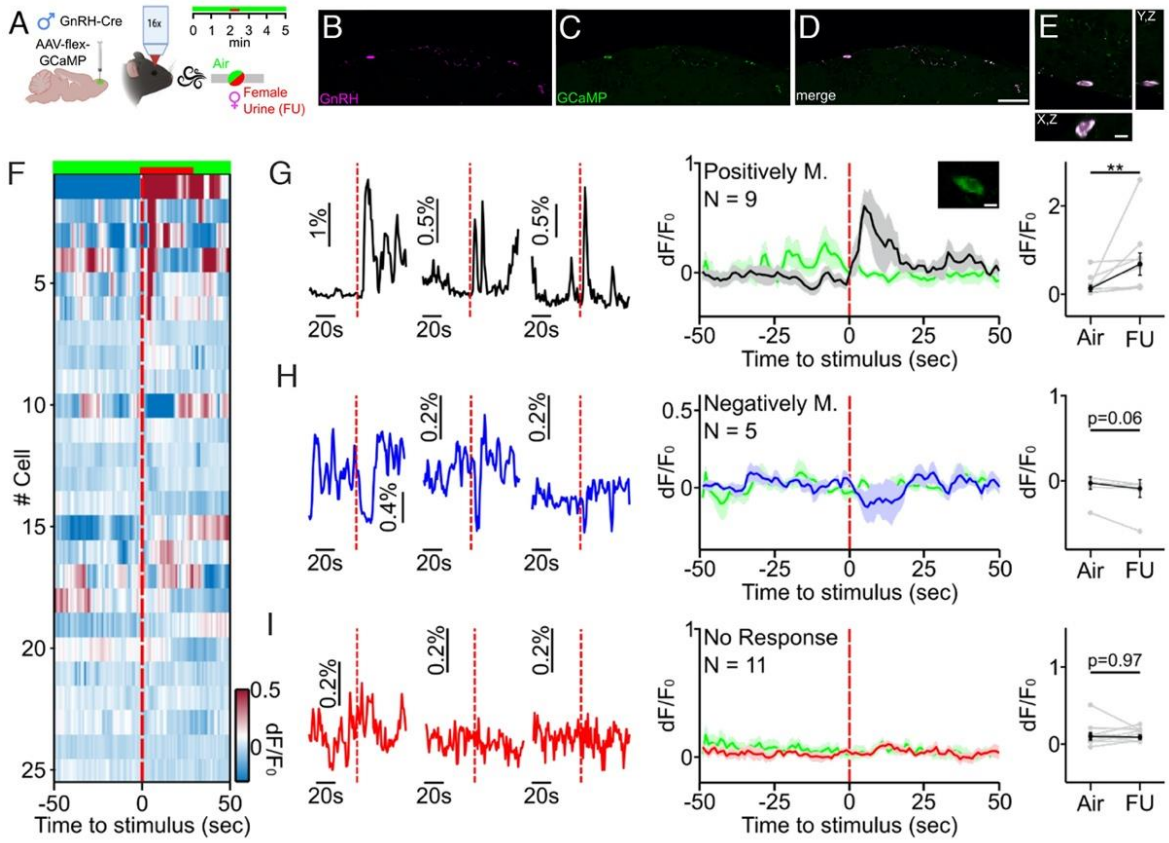


Figure 6: Female smell activates OB GnRH neurons. (A) Schematic of the experimental paradigm for *in vivo* two-photon functional imaging of GnRH-positive neurons during animal exposure to female urine (FU; N = 8, 12–16 weeks-old males). **(B-E)** Single plane confocal images showing (C) a GCaMP expressing neuron and processes (B) immunostained for GnRH. Merged images are shown in D. Scale bar in D = 50 µm and applies to B-C. **(E-F)** Higher magnification confocal images and reslices of the neuron shown in B-D. Scale bar in **E** = 10 µm. **(F)** Average fluorescence profiles of all recorded cells (N = 25). Green line on top indicates fresh airflow; red line on top indicates female urine odor presentation. Dashed red line indicates the stimulus onset. λ=920nm; power: 20-40 mW, depth: 30-60µm. **(G)** Left: Representative calcium traces from recorded GCaMP-expressing cells positively modulated by exposure to female urine. Vertical scale bars indicate dF/F_0, red line indicates stimulus onset. Centre: Average response profiles across all positively modulated neurons in presence of fresh air (green) or female urine (black). Shaded area indicates the standard error. Red dashed line indicates the stimulus onset. Image shows a representative GnRH cell expressing GCaMP and imaged under the two-photon microscope (scale bar: 10 µm). Right: Quantification of the peak dF/F_0 in the 10 seconds after the stimulus onset for positively modulated neurons. Grey dots and lines indicate individual cells, black dots and line represent the median and the associated standard error. **(H)** Same as G but for negatively (blue) modulated cells. **(I)** Same as G but for not responsive (red) cells.

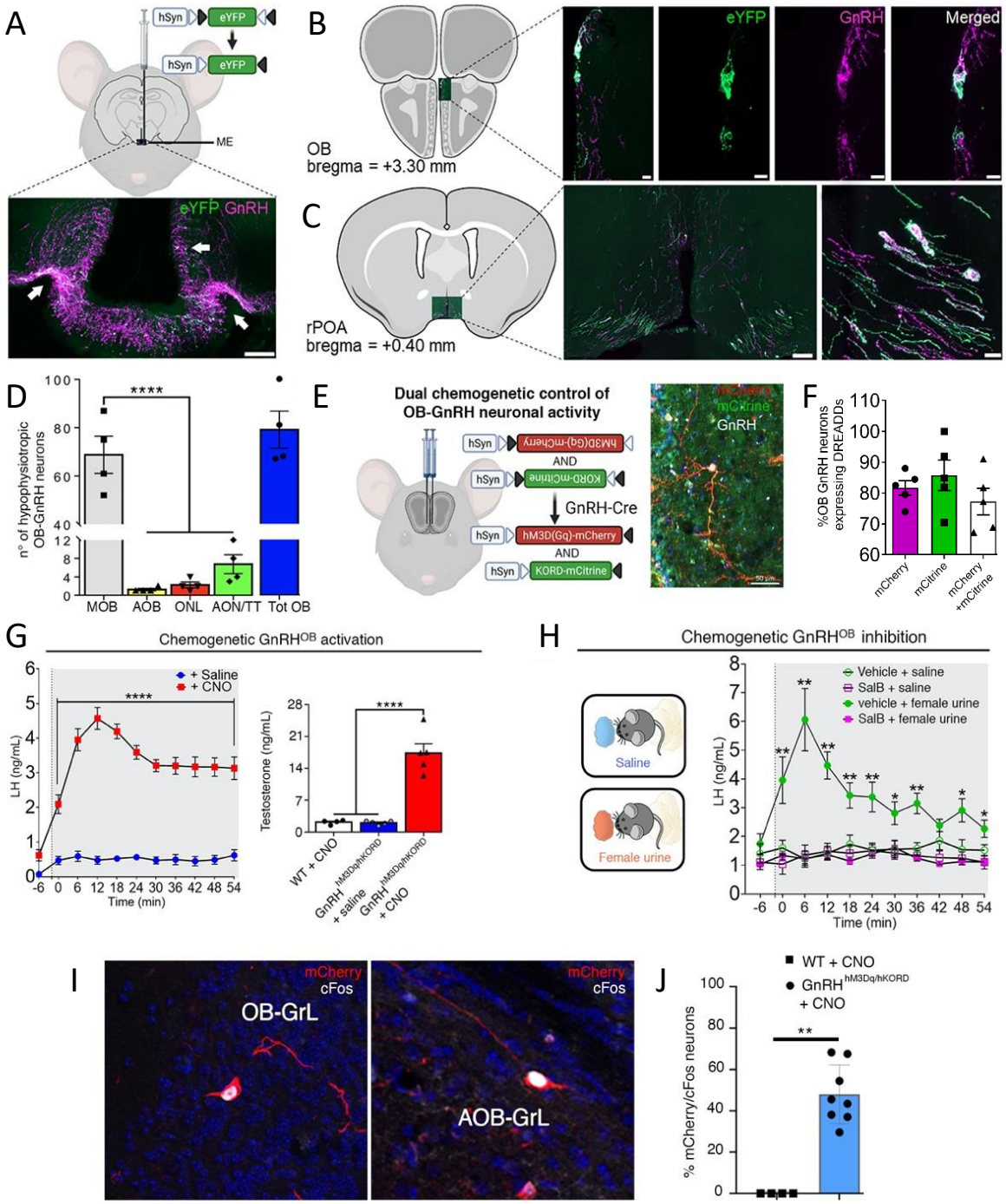
OB GnRH neurons are hypophysiotropic and mediate gonadotropin-release in males in response to female urine

Hypophysiotropic GnRH neurons are known to be mostly located in septal and hypothalamic areas from where they send long projections to the ME and secrete GnRH to coordinate LH release by the pituitary gland (Herbison, 2015; Wray and Hoffman, 1986). In order to estimate the fraction of OB GnRH neurons directly projecting to the ME, we stereotaxically delivered into the ME of *Gnrh1*$^{cre/wt}$ male mice a Cre-dependent AAV9, with anterograde and retrograde transport capabilities, encoding an enhanced yellow fluorescent protein (eYFP) and we confirmed colocalization of GnRH- and eYFP-expressing neuronal terminals innervating the ME (**Figure 7A**). We also showed the presence of GnRH-immunoreactive cells and fibers expressing eYFP in the OB (**Figure 7B**) as well in the POA (**Figure 7C**). We showed that nearly 80 OB GnRH neurons are putative hypophysiotropic cells (**Figure 7D**) and that most of those neurons are located in the MOB (68.75 ± 7.71), while a smaller fraction resides in the AOB (1.25 ± 0.25), ONL (2.25 ± 0.63), and AON/TT (6.75 ± 2.06) (**Figure 7D**).

Previous evidence showed that urinary compounds of opposite-sex modulate gonadotropin and gonadal sex hormone secretion in males (Maruniak and Bronson, 1976) and that POA GnRH neurons are activated after exposure to opposite-sex smell (Yoon et al., 2005). We hence aimed to modulate the activity of these cells *in vivo* using Cre-dependent designer receptors exclusively activated by designer drugs (DREADD) to unveil the functional role of OB GnRH neurons in GnRH-induced LH secretion in male mice. To this end, we reciprocally modulated OB GnRH neurons by co-infusing two Cre-dependent AAVs encoding hM3D(Gq) (AAV9-hSyn-DIO-hM3D(Gq-)mCherry), an activator DREADD having clozapine-N-oxide (CNO) as its ligand, and hKORD (AAV8-hSyn-DIO-hKORD-IRES-mCitrine), an inhibitory Gi-coupled kappa-opioid receptor DREADD activated by salvinorin B (SALB) (Vardy et al., 2015), into the OB of *Gnrh1*$^{cre/wt}$ male mice (GnRH$^{hM3Dq/hKORD}$; **Figure 7E**). Approximately 80% of GnRH$^{hM3Dq/hKORD}$ in the OB expressed both mCherry and mCitrine (**Figure 7F**). To investigate whether GnRH neurons can directly elicit a reproductive neuroendocrine response, we measured serum LH pulsatility by serial blood tail sampling in GnRH$^{hM3Dq/hKORD}$ male mice after administration of the chemogenetic drugs. Chemogenetic activation of OB GnRH neurons with

1 mg/kg CNO i.p. injection triggered a four-fold increase in LH secretion compared with baseline levels, whereas we did not observe any changes when the same group was injected with a saline vehicle (**Figure 7G**). This LH surge displayed peak levels around 12 minutes after CNO administration and was followed by a steady plateau over the remaining hormonal assessment time (**Figure 7G**). We also confirmed the brain activity signature by screening the expression of cFos, as a proxy of neuronal activation, in OB GnRH neurons. While we did not detect any double-stained mCherry/cFos in OB GnRH neurons of WT male mice injected with CNO, we found that approximately 50% of virally transduced GnRH neurons in the OB of $GnRH^{hM3Dq/hKORD}$ mice express cFos following CNO treatment (**Figure 7I, J**). Upon sexual encounters, male rodents typically exhibit a reflexive testosterone release, which is likely to be mediated by GnRH neuron activation accompanied by the upregulation of LH-mediated testicular steroidogenesis (Nyby, 2008). We found that chemogenetic activation of OB GnRH neurons elicited a marked increase in circulating testosterone levels in $GnRH^{hM3Dq/hKORD}$ male mice administered with CNO as compared with either WT mice injected with CNO or $GnRH^{hM3Dq/hKORD}$ male mice injected with vehicle (**Figure 7G**). Male mice are preferentially attracted to ovary-intact females in proestrus and estrus stages (Ingersoll and Weinhold, 1987). Thus, to test the requirement of OB GnRH neurons for pheromonal-mediated LH response, $GnRH^{hM3Dq/hKORD}$ male mice were treated with SALB or vehicle, and exposed to either urine collected from estrus females or to saline (**Figure 7H**). While female odor elicited a sharp increase in LH release over nearly one hour following vehicle administration, chemogenetic silencing of OB GnRH neurons with SALB completely blunted this gonadotropic response (**Figure 7H**).

These results demonstrate that OB GnRH neurons are mostly hypophysiotropic and mediate LH and testosterone secretion in male mice following the exposure to opposite-sex olfactory cues.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KORD-mCitrine
GnRH-Cre
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mCherry
mCitrine
mCherry +mCitrine
G
Chemogenetic GnRHOB activation
+ Saline
+ CNO
****
LH (ng/mL)
Time (min)
Testosterone (ng/mL)
****
WT + CNO
GnRHhM3Dq/hKORD + saline
GnRHhM3Dq/hKORD + CNO
H
Chemogenetic GnRHOB inhibition
Saline
Female urine
Vehicle + saline
SalB + saline
vehicle + female urine
SalB + female urine
LH (ng/mL)
Time (min)
I
mCherry
cFos
OB-GrL
mCherry
cFos
AOB-GrL
J
WT + CNO
GnRHhM3Dq/hKORD + CNO
**
% mCherry/cFos neurons

Figure 7: OB GnRH neurons mediate LH and testosterone release in response to female urinary odors in male mice. (A) Schematic of the experimental setup for the ME eYFP stereotaxic injections and representative coronal section of the ME showing GnRH (magenta) and eYFP (green) expression. **(B, C)** Representative coronal sections of (B) the OB and (C) the rPOA depicting the expression of GnRH (magenta) and eYFP (green). Colocalization between the GnRH and the eYFP is shown in white. **(D)** Number and distribution of hypophysiotropic OB GnRH neurons in male mice (N = 4; 12 weeks-old males). **(E)** Schematic representation of the experimental design of the dual chemogenetic control of OB GnRH neuronal activity. **(F)** Percetage of mCherry- and mCitrine-expressing, and the colocalization of both reporters in OB GnRH neurons in male mice (N = 5). **(G)** LH and testosterone levels after chemogenetic OB GnRH activation. CNO injections are performed 1 min before T_0 for LH pulsatility and 1h30 min before blood collection for testosterone (N = 8; 16-20 weeks-old males). **(H)** LH levels after chemogenetic OB GnRH inhibition. Salvinorin B injections are performed 1 min before opposite sex-smell exposure (N = 12; 16-20 weeks-old males). **(I)** Representative coronal sections of the MOB and AOB showing mCherry (red) and cFos (white) expression after CNO injection. **(J)** Percentage of mCherry+ cells expressing cFos after CNO injection. $GnRH^{hM3Dq/hKORD}$ male mice N = 8; WT animals N = 4; 16-20 weeks-old males. Two-way ANOVA; Tuckey's multiple comparisons test; Unpaired Mann-Whitney test; values are represented as mean ± SEM; * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$. At least 3 experimental replicates were performed for each of the above experiments. OB-GrL: main olfactory bulb granular cell layer, AOB-GrL: accessory olfactory bulb granular cell layer, CNO: Clozapine-N-Oxide.

OB GnRH neurons activate rPOA GnRH neurons

Due to the revealed connectivity of OB GnRH neurons with rPOA (**Figure 2G-M**) and to the demonstration that OB GnRH neurons' acute activation drives LH release, we wondered whether these neurons can also communicate with the hypothalamic GnRH neuronal population located in the rPOA. In order to test this hypothesis, we bilaterally delivered the Cre-dependent AAV9-hM3D(Gq)-mCherry into the OB of *Gnrh1*$^{cre/wt}$*::Gnrh1*^{gfp} male mice and we established the anatomical and functional connectivity between these neuronal populations using immunofluorescence and electrophysiological recordings (**Figure 8**). Histological evaluation tissue-cleared 200-µm thick brain slice preparations containing the rPOA confirmed that hypothalamic GnRH cell bodies do not express mCherry (**Figure 8A-D**). However, we observed mCherry-ir fibers crossing this territory and enwrapping GnRH cell bodies and dendrites (**Figure 8A-E**). Using confocal microscopy and 3D-reconstruction analysis, we identified contact spots (**Figure 8F**, white spots, arrows) between OB GnRH mCherry+ fibers both on the soma and along the primary dendrite of POA GnRH neurons.

We then performed whole-cell current-clamp recordings of rPOA GnRH-GFP neurons before, during and after bath application of 1 µM CNO. In the absence of CNO, most POA GnRH neurons exhibited typical tonic firing (**Figure 8G**). Bath application of 1µM CNO triggered a robust and sustained increase in the spontaneous action potential firing rate of POA GnRH neurons as compared with basal conditions ($P < 0.01$; **Figure 8G, H**). This was accompanied by an increase in the resting membrane potential of POA GnRH neurons compared with basal conditions ($P < 0.01$; **Figure 8I**). Taken together, these data show that activation of OB GnRH neurons modulates, either directly (through direct connections) or indirectly (through cell-network effect), the neuronal activity of POA GnRH neurons, which may be critical for olfactory-mediated gonadotropin secretion.

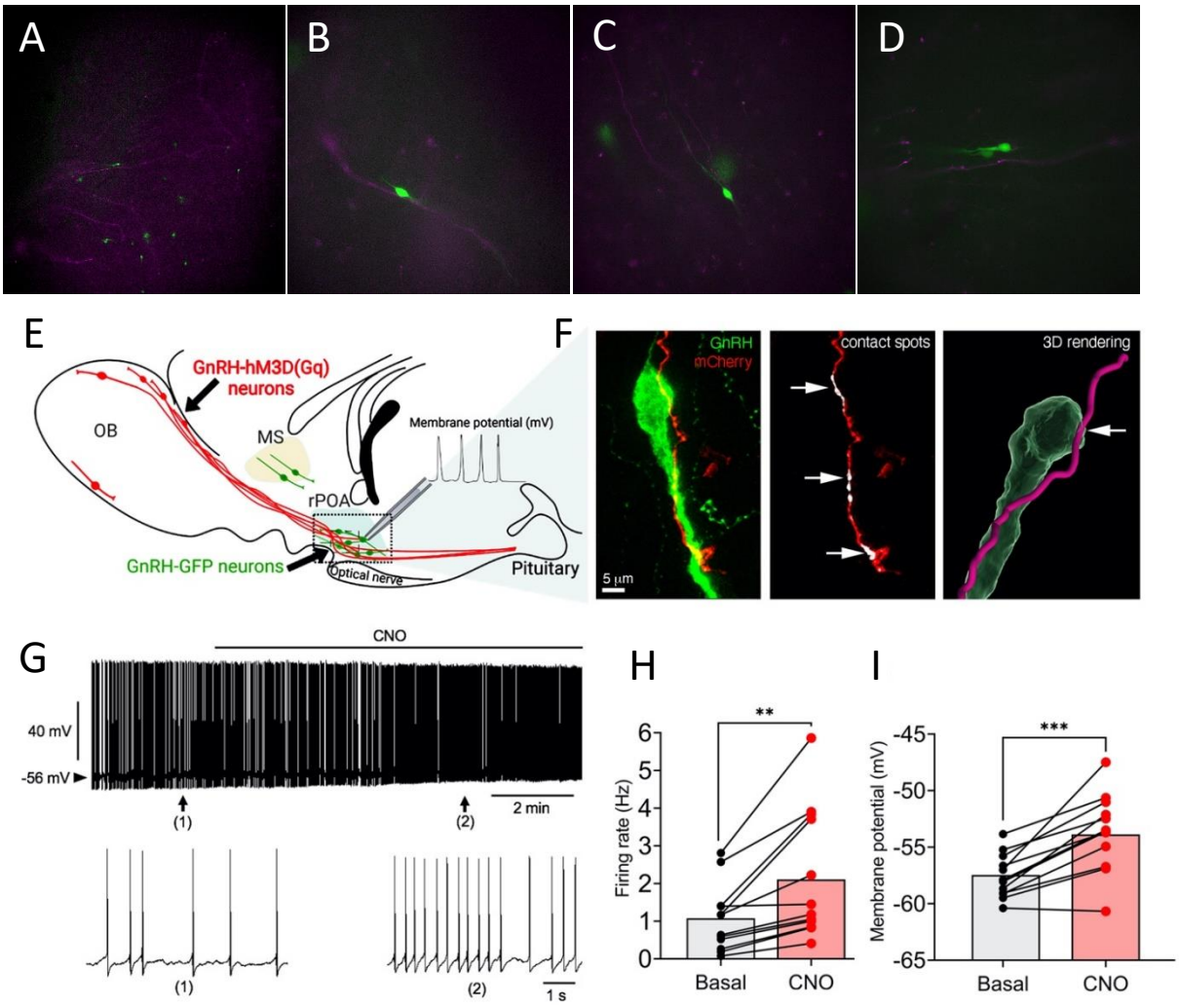

Figure 8: OB GnRH neurons communicate with POA GnRH neurons. (A) Schematic representing the electrophysiology experimental design. $GnRH^{OB}$ neurons are activated by 1µM bath application of CNO and POA GnRH neuronal activity is recorded using patch-clamp. Confocal images of 200µm thick sections and 3D rendering showing GnRH (green) and m-Cherry (red) labelling. White arrows highlight the contact points between OB GnRH and POA GnRH neurons. Scale bar: 5µm. **(B)** Whole-cell current-clamp recording showing the typical spontaneous burst firing of a POA GnRH neuron before and during bath application of CNO. Bottom traces show expansions of the recording at the indicated time points 1 and 2. **(C)** Firing rate (Hz) and **(D)** membrane potential (mV) of POA GnRH neurons increase after bath application of 1 µM CNO (N = 6 experimental replicates; n = 12 recorded cells; 12-16 weeks-old males; $^{**}P < 0.01$; $^{***}P < 0.001$; non-parametric paired Wilcoxon signed rank test; values are represented as means ± SEM).

OB GnRH neurons are required for opposite sex-odor discrimination

Previous results demonstrated the role of GnRH in olfactory preference. With the aim of unraveling the role of OB GnRH neurons we next sought to investigate whether OB GnRH neuronal activation and inhibition modulate olfactory preference to facilitate sexual behavior. To this aim, we reciprocally modulated OB GnRH neuronal activity by co-infecting OB GnRH neurons of sexually naive *Gnrh1*$^{cre/wt}$ male mice with both AAV9-hM3D(Gq)-mCherry and AAV9-KORD-mCitrine DREADDs (**Figure 9A**). Four weeks after the viral delivery, we subjected these animals to an olfactory preference test in which we evaluated the responses to urine from isolated stud male and estrus female mice while activating or inhibiting the activity of OB GnRH neurons in the same animals (**Figure 9B**).

Results demonstrated that chemogenetic activation of OB GnRH neurons tend to increase olfactory preference toward opposite-sex odors as male mice spent more time exploring female urine when injected with 1 mg/kg CNO as compared with their basal preference (saline = 52.11 ± 4.05% vs. CNO = 66.11 ± 3.07%; * $P < 0.05$; **Figure 9C**). The differences previously described were not attributable to a decrease of the time spent investigating both smells between saline or CNO injections (saline = 27.30 ± 4.088 sec vs. CNO = 32.99 ± 6.525 sec; ns $P > 0.05$; **Figure 9D**), an altered number of explorations, as male mice inspected either source of odor similarly (ns $P > 0.05$; **Figure 9E, F**), nor to the latency to reach them the first time (ns $P > 0.05$; **Figure 9G, H**).

Subsequently, a chemogenetic inhibition protocol was performed in the same animals described above using 10 mg/kg SALB. Novel male and female urine were used to test whether OB GnRH neurons are required for the display of olfactory preference. We discovered that inhibition of OB GnRH neurons robustly shifted olfactory preference, whereby SALB-treated male mice spent less time investigating female urine and more time investigating male urine, compared with vehicle-treated males (vehicle = 63.65 ± 5.07% vs. SALB = 30.01 ± 3.02%; *** $P < 0.001$; **Figure 9I**). The differences observed are not attributable to a modification of the investigation time since males spend the same amount of time investigation both odors treated with vehicle or salvinorin B (vehicle = 18.55 ± 7.407 vs. SALB = 9.412 ± 1.931; ns $P >$

0.05; **Figure 9J**). However, unlike previous observations, during OB GnRH inhibition we observed a significant decrease in the number of explorations toward female urine and not toward male urine (** $P < 0.01$; **Figure 9K-L**), despite a significant increase in latency to reach male scents (* $P < 0.05$; **Figure 9N**). The latency to reach female odor did not change between vehicle and Salvinorin B injections (ns $P > 0.05$; **Figure 9M**). Silencing OB GnRH neurons impact olfactory preference drive.

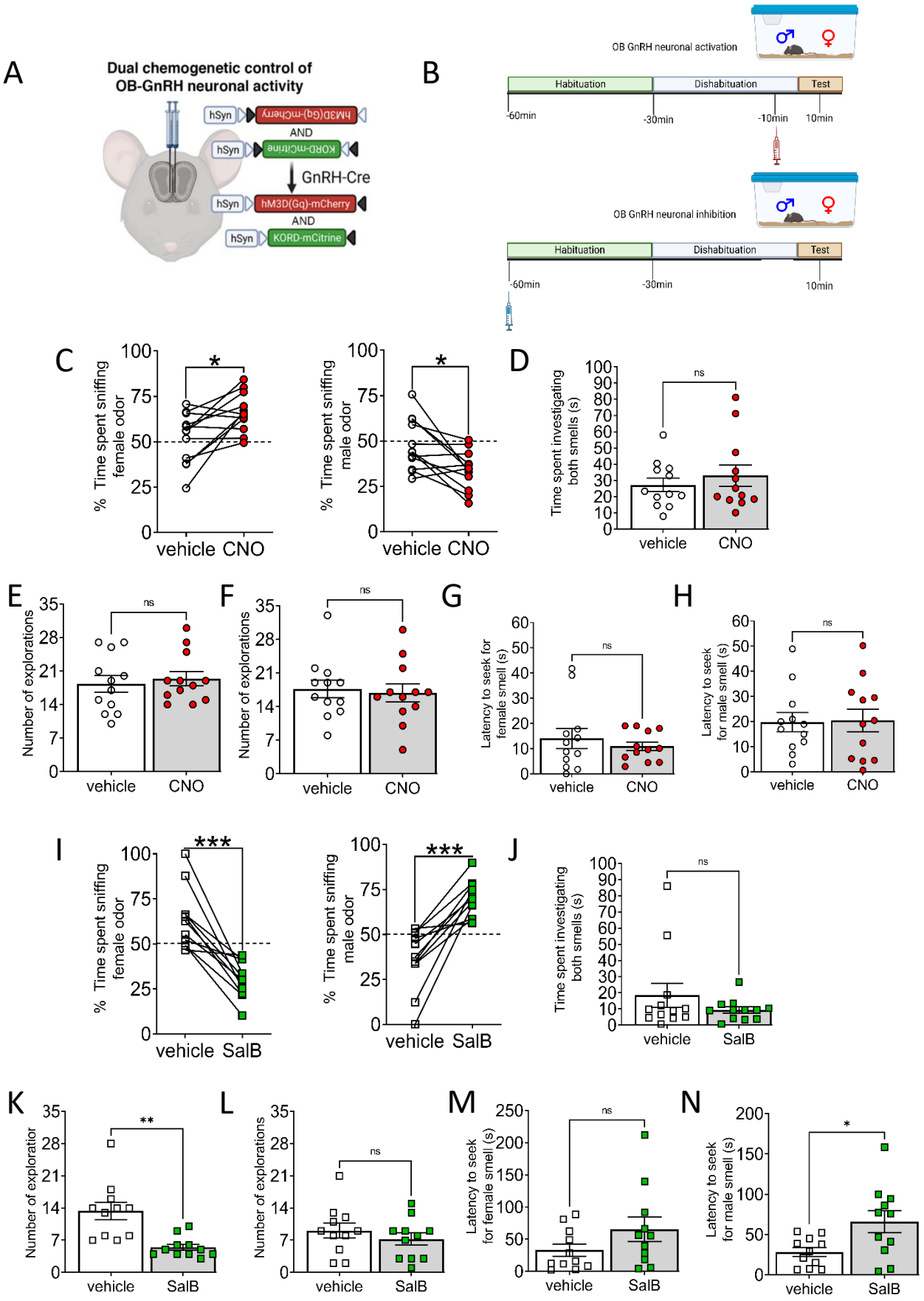
A
Dual chemogenetic control of
OB-GnRH neuronal activity
hSyn
hM3D(Gq)-mCherry
AND
hSyn
KORD-mCitrine
GnRH-Cre
hSyn
hM3D(Gq)-mCherry
AND
hSyn
KORD-mCitrine
B
OB GnRH neuronal activation
Habituation
Dishabituation
Test
-60min
-30min
-10min
10min
OB GnRH neuronal inhibition
Habituation
Dishabituation
Test
-60min
-30min
10min
C
% Time spent sniffing female odor
vehicle CNO
% Time spent sniffing male odor
vehicle CNO
D
Time spent investigating both smells (s)
vehicle
CNO
ns
E
Number of explorations
vehicle
CNO
ns
F
Number of explorations
vehicle
CNO
ns
G
Latency to seek for female smell (s)
vehicle
CNO
ns
H
Latency to seek for male smell (s)
vehicle
CNO
ns
I
% Time spent sniffing female odor
vehicle SalB
% Time spent sniffing male odor
vehicle SalB
J
Time spent investigating both smells (s)
vehicle
SalB
ns
K
Number of explorations
vehicle
SalB
L
Number of explorations
vehicle
SalB
ns
M
Latency to seek for female smell (s)
vehicle
SalB
ns
N
Latency to seek for male smell (s)
vehicle
SalB

Figure 9: OB GnRH neurons drive olfactory preference in male mice. (A) Schematic of the experimental design of the dual chemogenetic control of OB GnRH neuronal activity. **(B)** Schematic of the experimental workflow. CNO injection was performed 10 min before the test and Salvinorin B injection 1 hour before. **(C)** Percentage of time spent investigating female and male odors during chemogenetic OB GnRH neuronal activation. **(D)** Total time spent investigating both odors after saline or CNO injections during the olfactory preference test. **(E-F)** Number of explorations towards female (E) or male (F) smell. **(G-H)** Latency to explore female (G) and male (H) smell after OB GnRH chemogenetic activation. **(I)** Percentage of time spent investigating female and male odors during chemogenetic OB GnRH neuronal inhibition. **(J)** Total time spent investigating both odors after DMSO or SalB injections during the olfactory preference test. **(K-L)** Number of explorations towards female (K) or male (L) smell. **(M-N)** Latency to explore female (M) and male (N) smell after OB GnRH chemogenetic inhibition. Chemogenetic activation N = 12, chemogenetic inhibition N = 11; 12-16 weeks-old males; non-parametric paired Wilcoxon signed rank test. Values are represented as means ± SEM. ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

To analyze the specific role of OB GnRH neurons in olfactory mate preference, we used a genetic strategy to specifically ablate these neuronal population by stereotaxically injecting an adeno-associated virus encoding a Cre recombinase-dependent caspase 3 (AAV9-flex-taCasp3-TEVp virus) bilaterally into the OB of mice expressing Cre in GnRH neurons (*Gnrh1*$^{cre/wt}$ male mice, **Figure 10A**). Caspase 3 kills the Cre-expressing cells by inducing apoptosis. GnRH neurons in the OB were unaffected in control animals (*Gnrh1*$^{wt/wt}$) but they were significantly reduced by 40% in *Gnrh1*$^{cre/wt}$ mice 2 weeks following viral delivery (**Figure 10B**).

We then tested whether the taCasp3-encoding AAV targeted to the OB diffused to and ablated GnRH cells in distant hypothalamic regions (POA). This analysis revealed no difference in GnRH cell number between control animals and viral-infected animals (**Figure 10C**), indicating that the taCasp3-mediated ablation is restricted to the proximity of the injection site.

Subsequently, we subjected the mice to the same behavioral paradigms described above (**Figure 9**) to test whether ablation of OB GnRH neurons impacted the display of olfactory preference. Our results showed that a partial ablation of OB GnRH neurons (~40%) is sufficient to alter olfactory preference of adult male mice as we observed that GnRH^{Casp3} males failed to show any female-directed preference as compared to control littermates (Mann-Whitney test; $P < 0.01$; **Figure 10D**).

Altogether, our chemogenetic and cell-specific ablation experiments support the role of OB GnRH neurons as a key component dictating sex-partner recognition.

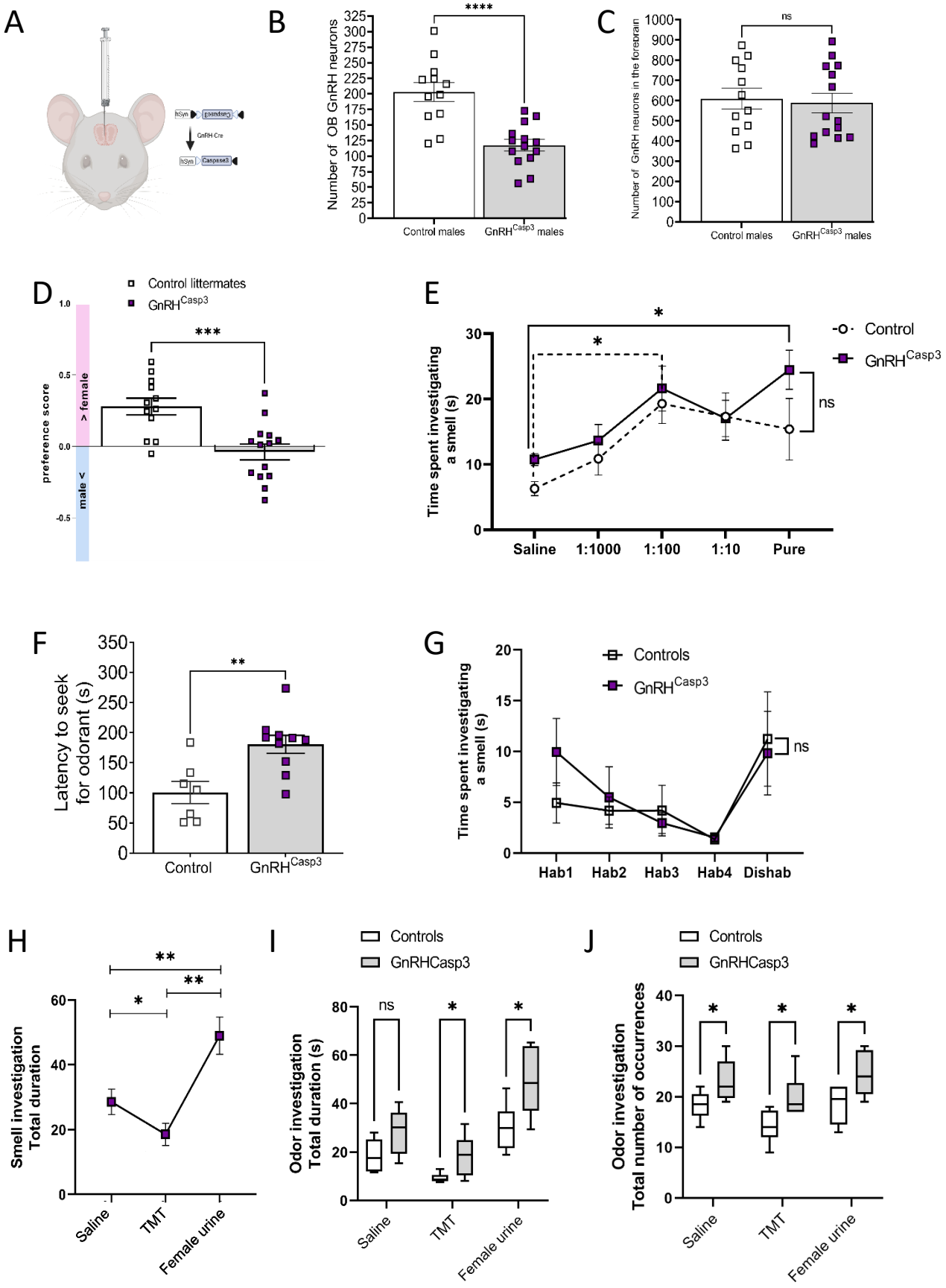
A
hSyn
GnRH-Cre
hSyn
B
Number of OB GnRH neurons
****
Control males
GnRHCasp3 males
C
Number of GnRH neurons in the forebrain
ns
Control males
GnRHCasp3 males
D
Control littermates
GnRHCasp3
***
preference score
> female
male <
E
Control
GnRHCasp3
Time spent investigating a smell (s)
*
*
ns
Saline
1:1000
1:100
1:10
Pure
F
Latency to seek for odorant (s)
**
Control
GnRHCasp3
G
Controls
GnRHCasp3
Time spent investigating a smell (s)
ns
Hab1
Hab2
Hab3
Hab4
Dishab
H
Smell investigation Total duration
**
**
*
Saline
TMT
Female urine
I
Controls
GnRHCasp3
Odor investigation Total duration (s)
ns
*
*
Saline
TMT
Female urine
J
Controls
GnRHCasp3
Odor investigation Total number of occurrences
*
*
*
Saline
TMT
Female urine

Figure 10: OB GnRH neurons are facilitating social olfactory cues detection. (A) Schematic representation of the bilateral AAV9-hSyn-DIO-Caspase3 stereotaxic injection in the OB in GnRH-Cre male mice. **(B, C)** Number of GnRH neurons in the OB (B) and hypothalamus (C) of control and $GnRH^{Casp3}$ male mice. Unpaired t-test. **(D)** Preference score of $GnRH^{Casp3}$ male mice and $GnRH^{wt/wt}$ littermates. Unpaired t-test. **(E)** Olfactory detection threshold test, using different range of diluted female urine, $GnRH^{Casp3}$ male mice vs $GnRH^{wt/wt}$ littermates. Two-way ANOVA. **(F)** Latency to seek for odorant during a buried odor seeking test in $GnRH^{Casp3}$ male mice and $GnRH^{wt/wt}$ littermates. Mann-Whitney test. **(G)** Habituation/dishabituation test using acetophenone as habituation odor and octanal as dishabituation odor. Two-way ANOVA. **(H-I)** Time spent investigating a smell in an open field arena and **(J)** total number of smell investigations during a 10 min test in $GnRH^{Casp3}$ male mice and $GnRH^{wt/wt}$ littermates. Two-way ANOVA. Values are represented as means ± SEM. N = 6 control mice *Gnrh1*$^{wt/wt}$ injected with an AAV9-hSyn-DIO-Caspase3 virus in the OB; N = 8 $GnRH^{Casp3}$ male mice injected with an AAV9-hSyn-DIO-Caspase3 virus in the OB, 12-16 weeks-old males. ns $P > 0.05$; * $P < 0.05$; ** $P < 0,01$; *** $P < 0,001$; **** $P < 0,0001$.

GnRH facilitate social olfactory cues detection

To analyze in more details the role of OB GnRH neurons in odor information processing, we used the GnRH^{Casp3} male mice and performed multiple olfactory tests with social and non-social odors (**Figure 10**).

The olfactory threshold test showed that control mice successfully differentiate between saline and female urine in a 1:100 dilution. In contrast, GnRH^{Casp3} mice only exhibit increased interest in estrus female urine when it is presented in its pure form, rather than at increasing dilution levels (Two-way ANOVA; * $P < 0.05$; N = 6 *Gnrh1*$^{wt/wt}$; N = 6 GnRH^{Casp3}; **Figure 10E**). These results suggest that OB GnRH neuronal ablation impacts social olfactory cues detection.

To assess whether odor seeking motivation was affected in OB GnRH genetically ablated animals, we conducted the buried odor seeking test. During the test, we buried a filter paper with pure estrus female urine under bedding in a random corner of the arena and monitored the latency to reach the filter paper by the mice. Our data showed that GnRH^{Casp3} male mice take significantly longer time to reach the odor compared to their control littermates. These findings suggest that OB GnRH neurons play a role in driving motivation to seek out opposite-sex smells (Mann-Whitney test; 79.74 ± 23.63; P = 0.0068; N = 7 *Gnrh1*$^{wt/wt}$; N = 10 GnRH^{Casp3}; **Figure 10F**).

We aimed to assess the importance of OB GnRH neurons in the detection of non-social olfactory cues. To do so, we conducted a habituation-dishabituation test using acetophenone and octanal. In this test, the same scent was presented to the mouse four times, followed by a different scent on the fifth presentation. Our findings indicate that no differences were detected in either the habituation or dishabituation phase between the two groups, suggesting that OB GnRH neurons are not involved in the detection of non-social olfactory cues (two-way ANOVA; ns $P > 0.05$; N= 6 *Gnrh1*$^{wt/wt}$; N = 6 GnRH^{Casp3}; **Figure 10G**).

To further investigate the discrimination of social odors, we assessed the ability of GnRH^{Casp3} male mice to differentiate between various social odors, including aversive fox urine stimuli. Our experiments found that GnRH^{Casp3} male mice spent significantly more time investigating estrus female urine than saline, and significantly less time investigating fox urine (TMT) than saline. This demonstrates that GnRH^{Casp3} male mice are capable of discriminating

between different social odors (two-way ANOVA; * $P < 0.05$; ** $P < 0.01$; N= 6 *Gnrh1*$^{wt/wt}$; N = 6 GnRH^{Casp3}; **Figure 10H**). In addition, during this test, GnRH^{Casp3} male mice spend a significantly greater amount of time investigating TMT and female odors compared to their control littermates (two-way ANOVA; * $P < 0.05$; N= 6 *Gnrh1*$^{wt/wt}$; N = 6 GnRH^{Casp3}; **Figure 10I**) and GnRH^{Casp3} males investigate the smells (saline, TMT and female urine) a significantly higher total number of time as compared with Gnrh1$^{wt/wt}$ mice (two-way ANOVA; * $P < 0.05$; N= 6 *Gnrh1*$^{wt/wt}$; N = 6 GnRH^{Casp3}; **Figure 10J**). These findings indicate that GnRH^{Casp3} male mice differenciate between neutral, aversive and appetitive odors, as well as control males. However, to get the same information, GnRH^{Casp3} male mice spend more time exploring the smells, suggesting that OB GnRH neurons may be relevant to detect social smells but not to differenciate them.

OB GnRH neurons facilitate sexual behavior initiation in naïve male mice

To decipher whether OB GnRH neurons mediate interest or motivation to seek for a partner and drive sexual behavior, we performed the mate preference test in a tree-chamber arena and the copulatory behavior test (**Figure 11**). During the mate preference test, a male and a female mouse were placed at the opposite sides of the arena. The male was isolated two weeks before the test, and the ovariectomized female mouse received estrogen and progesterone treatment to mimic the estrus phase. The mate preference test revealed that GnRH^{Casp3} male mice spent the same amount of time as compared with control mice, in female side (**Figure 11A**), male side (**Figure 11B**) and center (**Figure 11C**) (Unpaired t-test; ns $P > 0.05$; N= 13 *Gnrh1*$^{wt/wt}$; N = 14 GnRH^{Casp3}; **Figure 11A-C**). The total number of occurrences in either female or male sides were not different between the two groups (Unpaired t-test; ns $P > 0.05$; N= 13 *Gnrh1*$^{wt/wt}$; N = 14 GnRH^{Casp3}; **Figure 11D-E**). The OB GnRH neurons are not involved in opposite-sex conspecific recognition.

Then, we performed the copulatory behavioral test. During this test the female is placed in the male cage for 30 minutes. Naïve GnRH^{Casp3} male mice and controls have been trained during three different sessions before performing the final test. During the first training

session, the number of mounts is significantly lower in naïve GnRH^{Casp3} male mice than in naïve *Gnrh1*$^{wt/wt}$ male mice (Two-way ANOVA; * $P < 0.05$; N= 6 *Gnrh1*$^{wt/wt}$; N = 6 GnRH^{Casp3}; **Figure 11F**). Despite this difference, the number of mounts is not significantly different between the two groups during the two other learning sessions (Two-way ANOVA; ns $P > 0.05$; N= 6 *Gnrh1*$^{wt/wt}$; N = 6 GnRH^{Casp3}; **Figure 11F**) and during the sexual behavior test (Unpaired t-test; ns $P > 0.05$; N= 6 *Gnrh1*$^{wt/wt}$; N = 6 GnRH^{Casp3}; **Figure 11G**). During the test, the latency to first mount and the number of intromissions are not significantly different between the GnRH^{Casp3} and the *Gnrh1*$^{wt/wt}$ male mice (Unpaired t-test; ns $P > 0.05$; N= 6 *Gnrh1*$^{wt/wt}$; N = 6 GnRH^{Casp3}; **Figure 11H-I**).

Finally, we measured LH pulsatility after saline or pure female urine exposure in GnRH^{Casp3} and control male mice. Results show that 40% ablation of OB GnRH neurons is not sufficient to significantly decrease LH levels after estrus female urine exposure in GnRH^{Casp3} male mice as compared to *Gnrh1*$^{wt/wt}$ male mice (**Figure 12**).

To conclude on this part, these results showed that OB GnRH neurons facilitate social cues detection. This detection elicits interest towards opposite-sex smell and facilitates the initiation of sexual behavior in naïve males but does not modulate sexual behavior in experienced males.

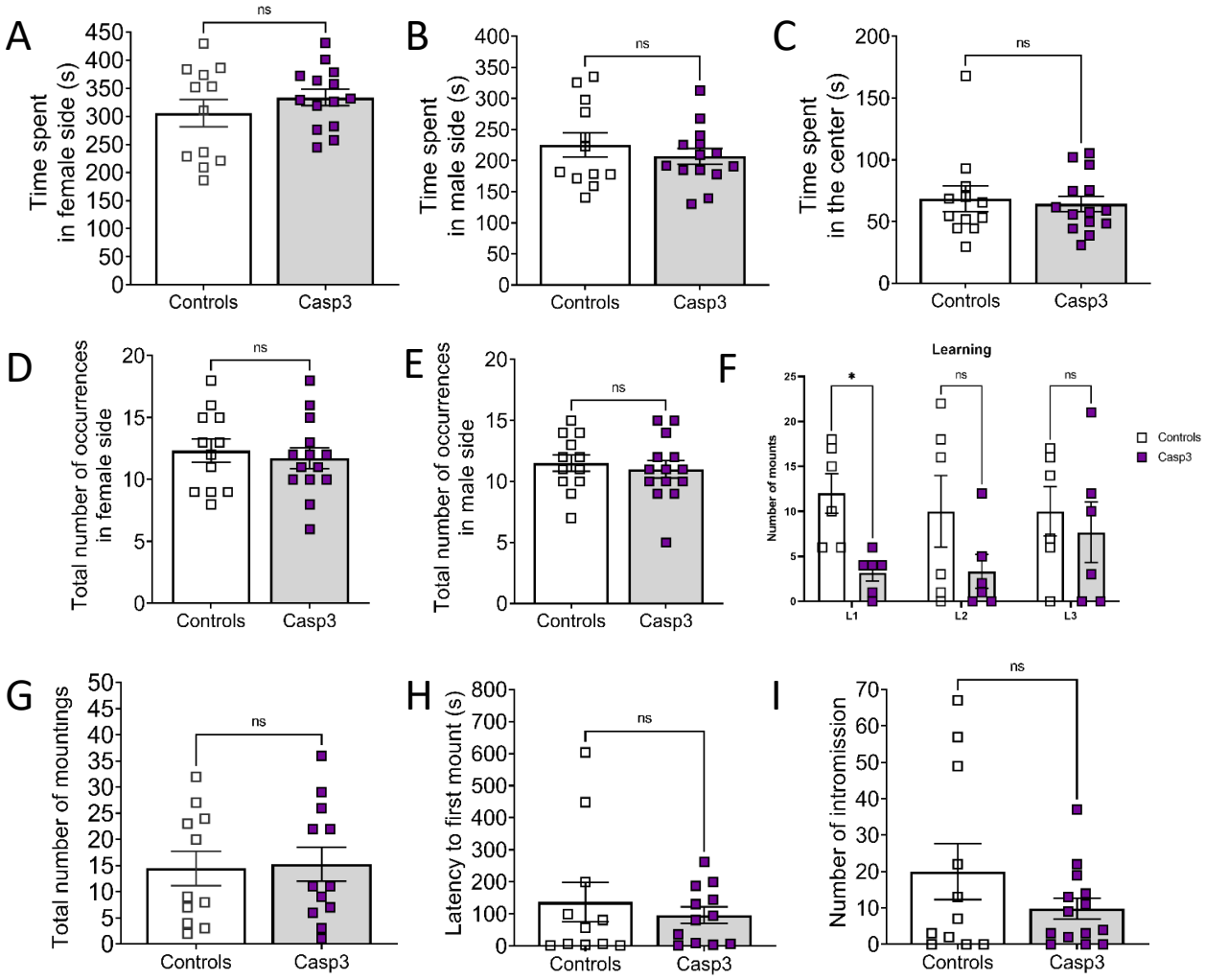

Figure 11: OB GnRH neurons are not driving mating. (A-C) Time spent in female (A), male (B), or center (C) of the tree-chambers arena during a mate preference test. **(D-E)** Number of entrances in the female (D) and male (E) sides during the tree-chambers arena during a mate preference test. **(F)** Number of mounts during the tree learning sessions preceding the copulatory test. **(G)** Number of mounts during the sexual behavior test. **(H)** Latency to first mount during the sexual behavior test. **(I)** Number of intromissions during the sexual behavior test. Unpaired-t test and Two-way ANOVA. N = 12 control mice *Gnrh1*$^{wt/wt}$ injected with an AAV9-hSyn-DIO-Caspase3 virus in the OB; N = 14 GnRH^{Casp3} male mice injected with an AAV9-hSyn-DIO-Caspase3 virus in the OB, 12-16 weeks-old males. (F) N = 6 *Gnrh1*$^{wt/wt}$ and N = 6 GnRH^{Casp3}. Values are represented as means ± SEM. ns $P > 0.05$; * $P < 0.05$; ** $P < 0,01$.

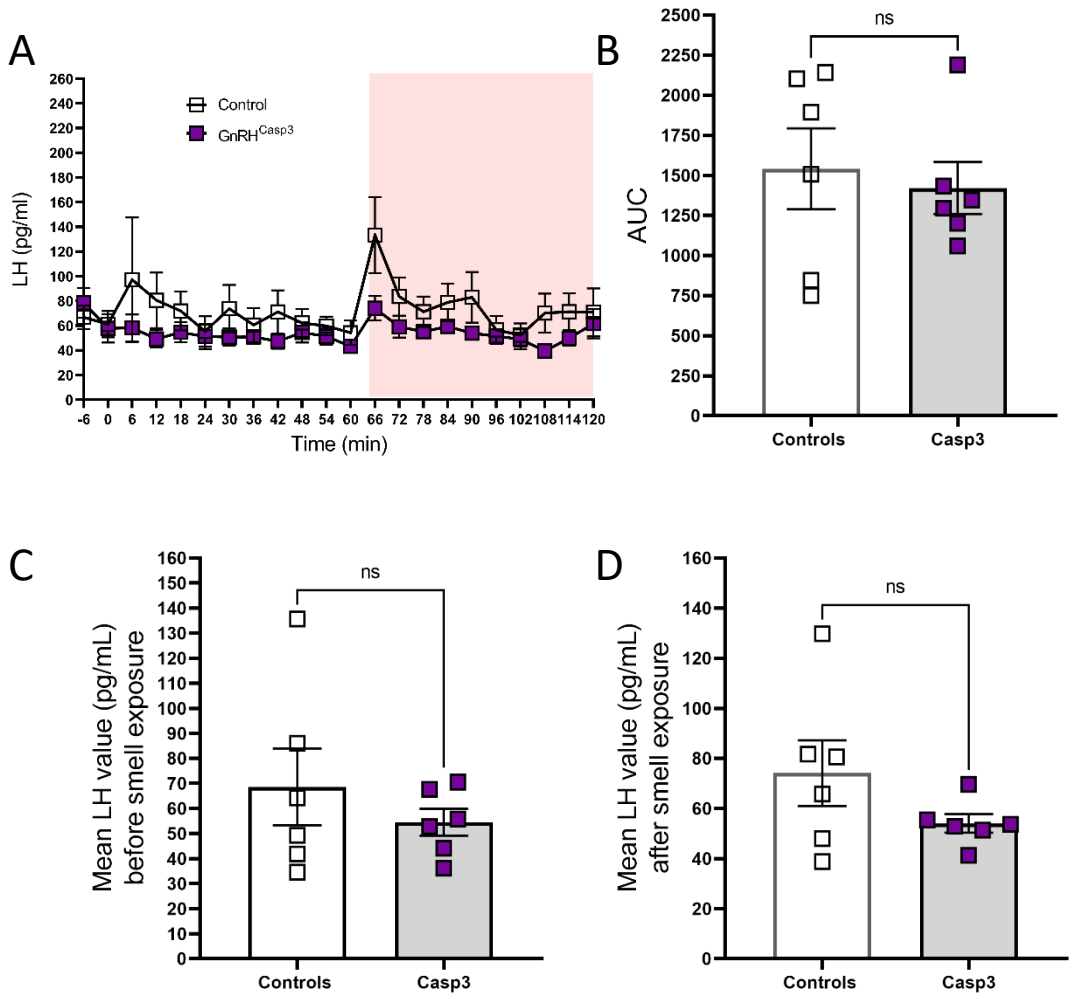

Figure 12: 40% OB GnRH neuronal ablation is not sufficient to alter LH secretion in response to opposite-sex odor exposure. (A) LH levels after injection of an AAV9-hSyn-DIO-Caspase3 virus in the OB of *Gnrh1*$^{cre/wt}$ and *Gnrh1*$^{wt/wt}$ male mice. Mice were exposed to opposite-sex pure urine 1 min before T66. Two-way ANOVA. **(B)** AUC during LH pulsatility in controls and GnRH^{Casp3} male mice. Unpaired-t test. **(C)** Mean LH values before exposing male mice control littermates and GnRH^{Casp3} male mice to female urine. Unpaired-t test. **(D)** Mean LH value after control littermates and GnRH^{Casp3} male mice exposure to female urine. Unpaired-t test. N = 6 control mice injected with an AAV9-hSyn-DIO-Caspase3 virus in the OB; N = 6 GnRH^{Casp3} male mice injected with an AAV9-hSyn-DIO-Caspase3 virus in the OB, 12-16 weeks-old males. Values are represented as means ± SEM. ns $P > 0.05$.

The posterodorsal medial amygdala (pdMeA) is a downstream target of OB GnRH neurons driving opposite-sex odor preference and copulatory behavior

The medial amygdala (MeA) is involved in the control of many different innate emotional behaviors such as responses to predator, aggressive intruder or sexual partner (Keshavarzi et al., 2014; Kondo, 1992). Olfactory information collected by the VNO are transmitted to the AOB which is directly connected to the MeA (Dulac and Torello, 2003). Our aforementioned observations showed that GnRH-R expressing cells are located throughout the MOS and AOS pathways, including the MeA, with a high number of GnRH-R in the pdMeA in male mice (**Chapter 3, Figure 3 and 4**). We evaluated whether exposure to female urine could induce cFos expression in GnRH-R neurons located along the MOS and AOS pathways in GRIC/R26-τGFP male mice (**Figure 13**). Stimulated animals showed increased percentages of GnRH-R cells expressing cFos in the OB, aMeA, pdMeA and PC (Mann-Whitney test, total OB ctrl *vs* total OB stimulus, $P = 0.028$; aMeA water *vs.* aMeA female urine, $P = 0.028$; pdMeA water *vs.* pdMeA female urine, $P = 0.028$, PC water *vs* PC female urine, $P = 0.028$; **Figure 13A-G**). These data show that attraction to opposite-sex odor in males is mediated by GnRH, most likely via activation of GnRH-R cells distributed throughout the MOS and AOS.

Neuroanatomical observations of female odor-stimulated male mice revealed that within the pdMeA GnRH-positive fibers are located in proximity of cFos+ pdMeA GnRH-R expressing cells cells (**Figure 13H-O**). Next, we injected the cre-dependent inhibitory chemogenetic receptor hM4Di (AAV9-hM4D(Gi)-mCherry) into the pdMeA of *Gnrh1*$^{cre/wt}$ male mice to selectively infect the GnRH neurons projecting to the pdMeA (**Figure 13P**). Immunohistochemical analysis revealead that OB GnRH neurons sending projections to the MeA are mainly located within the AOB (**Figure 13Q**). Therefore, we hypothesized that OB GnRH neuronal inputs into the pdMeA may be required to drive opposite-sex odor preference and could modulate sexual behavior. To evaluate this point, male mice were injected with either the AAV9-hM4D(Gi)-mCherry virus or the AAV9-mCherry virus into the pdMeA. *In vivo* chemogenetic inhibition of the sub-population of OB GnRH neurons sending direct projections to the pdMeA significantly abrogated the olfactory preference of GnRH^{hM4Di} male mice toward the female urine and it shifted the olfactory preference in favor of the same-sex smell (Unpaired t-test; N=9

GnRH^{hM4Di}; **** $P < 0.0001$*;* **Figure 14A**). The olfactory preference did not change for the control group injected either with saline or CNO (Unpaired t-test; N=9 GnRH^{mcherry}; $P = 0.2223$*;* **Figure 14B**). In addition, we did not detect any difference in the number of explorations nor in the latency to seek for the female or male smell in GnRH^{hM4Di} male mice (Paired t-test; =9 GnRH^{hM4Di}; ns $P > 0.05$; **Figure 14C-F**), indicating that inhibition of OB GnRH neurons does not impact the motivation of these animals to seek for a novel odor.

Since we targeted the MeA, an area known to be involved in innate behaviors as previously described, we performed sexual copulatory behavior test on naïve males. Chemogenetic inhibition of OB GnRH neurons projecting to the pdMeA impaired the copulatory behavior of GnRH^{hM4Di} male mice, which showed a significant decrease in the number of mounts after CNO injections compared to vehicle injections (Paired t-test; N=9 GnRH^{hM4Di}; N= 9 GnRH^{mcherry}; $P = 0.0310$; **Figure 15A**), whereas they did not exhibit any changes in the latency to first mount (Paired t-test; N=9 GnRH^{hM4Di}; ns $P > 0.05$; **Figure 15B**). We confirmed these results by injecting GnRH$^{cre/wt}$ males with AA9-mCherry control virus and performed the same sexual behavioral test. Results did not show any significant differences regarding the number of mounts or the latency to first mount in the control group between saline and CNO injections (Paired t-test; N=9 GnRH^{mcherry}; ns $P > 0.05$; **Figure 15C-D**).

Because olfactory mate preference and reproductive behavior in mammals can be affected by anxiety and locomotor activity, we assessed these parameters in GnRH^{hM4Di} virally transduced with the inhibitory DREADD and GnRH^{mcherry} males. We employed the elevated plus-maze test (EPM) to measure anxiety in male mice (**Figure 15E-J**) and the open field arena with infrared tracking to measure locomotor activity (**Figure 16**). Our results show that inhibition of the OB GnRH neuron-to-pdMeA pathway does not affect any of the evaluated parameters in this commonly used behavioral tests in GnRH^{hM4Di} and GnRH^{mcherry} male mice (Paired t-test; N=9 GnRH^{hM4Di}; N=9 GnRH^{mcherry}; ns $P > 0.05$; **Figure 15E-J**) and suggest that these neurons are required for sex recognition and the display of mating with females in naïve male mice.

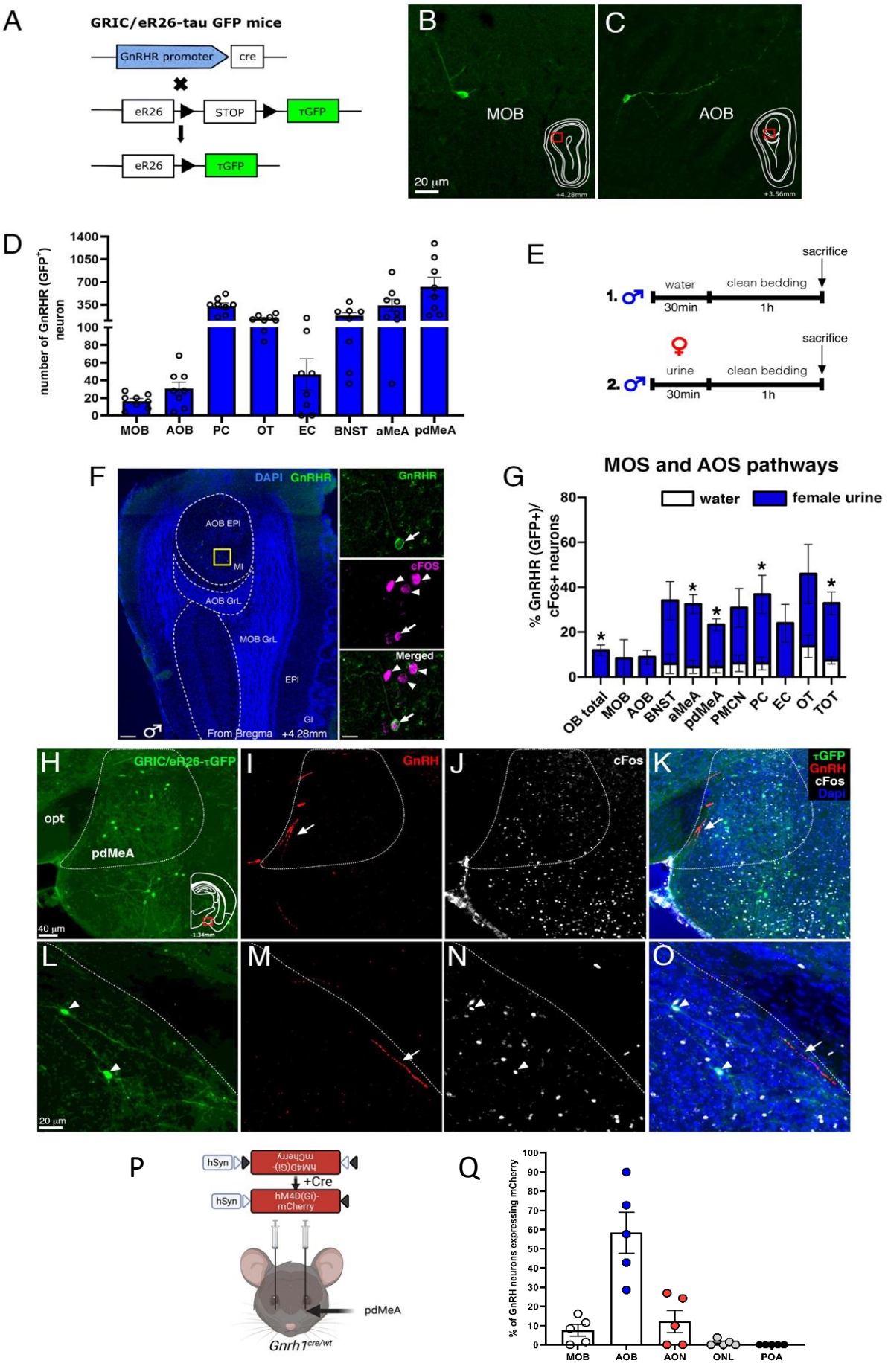
A
GRIC/eR26-tau GFP mice
GnRHR promoter
cre
eR26
STOP
τGFP
eR26
τGFP
B
MOB
20 µm
+4.28mm
C
AOB
+3.56mm
D
number of GnRHR (GFP+) neuron
1400
1050
700
350
100
80
60
40
20
0
MOB
AOB
PC
OT
EC
BNST
aMeA
pdMeA
E
sacrifice
1. ♂
water
clean bedding
30min
1h
sacrifice
♀
2. ♂
urine
clean bedding
30min
1h
F
DAPI GnRHR
AOB EPI
MI
AOB GrL
MOB GrL
EPI
GI
From Bregma +4.28mm
GnRHR
cFOS
Merged
G
MOS and AOS pathways
water
female urine
% GnRHR (GFP+)/ cFos+ neurons
80
60
40
20
0
OB total
MOB
AOB
BNST
aMeA
pdMeA
PMCN
PC
EC
OT
TOT
H
GRIC/eR26-τGFP
opt
pdMeA
40 µm
I
GnRH
J
cFos
K
τGFP
GnRH
cFos
Dapi
L
20 µm
M
N
O
P
hSyn
hM4D(Gi)-mCherry
+Cre
hSyn
hM4D(Gi)-mCherry
pdMeA
Gnrh1cre/wt
Q
% of GnRH neurons expressing mCherry
100
90
80
70
60
50
40
30
20
10
0
MOB
AOB
AON
ONL
POA

Figure 13: OB GnRH neurons are sending projections to the postero-dorsal part of the medial amygdala and exert their actions through the GnRH receptors found in the same area. (A) Genetic strategy to label GnRH-R-expressing neurons. **(B-C)** Confocal images of representative coronal sections of the **(B)** main olfactory bulb and **(C)** accessory olfactory bulb showing GnRH-R expressing neurons and processes immunostained for GFP (in green) in GRIC/eR26-ɾGFP mouse-line. White drawings depict OB sections and Bregma coordinates corresponding the sections represented in **B** and **C** and red boxes indicate the region from which the confocal images were taken. **(D)** Quantification of GnRH-R cells (GFP+) throughout the brain regions involved in olfactory processing (N = 8). Values are shown as mean ± SEM. **(E)** Experimental protocol: GRIC/eR26-ɾGFP male mice were exposed either to water (1) or to female-soiled bedding (2) for 30 minutes followed by a clean bedding exposure for 1 hour before sacrifice and cFos immunolabeling. **(F)** Coronal section of OB (at Bregma +4.28) labelled for GnRH-R (GFP, in green) and DAPI (in blue). Yellow inset in the AOB corresponds to high magnification images in the right-hand pictures depicting a double-labelled GnRH-R (green)-cFos+ (magenta) cell in the AOB mitral layer (Ml; arrow). **(G)** Percentage of GnRH-R cells co-expressing cFOS along the MOS and the AOS pathways after exposure to water or female urine. The values shown are mean ±SEM. GRIC/eR26-ɾGFP male mice exposed to water (N = 4; 12 weeks-old males) or to female-soiled bedding (N = 4; 12 weeks-old males); Mann-Whitney test. **(H-K)** Coronal section of the pd-MeA showing the expression of GRIC/eR26-tauGFP (green); GnRH (red); cFos (white) and merge respectively. The red box indicates the imaged area. **(L-O)** Higher magnification of the pd-MeA showing GRIC/eR26-tauGFP (green); GnRH (red); cFos (white) expression and merge respectively. **(P)** Schematic representation of the bilateral AAV-hSyn-DIO-hM4D(Gi)-mCherry stereotaxic injection in the pdMeA. **(Q)** Percentage of OB GnRH neurons co-expressing mCherry after AAV9-hSyn-DIO-hM4D(Gi)-mCherry stereotaxic injections in the pdMeA. * $P < 0.05$. Scale bars: **(B-C)** 20 µm; **(F)** 100 µm left image; 20 µm right images; **(H-K)** 40 µm; **(L-O)** 20 µm. Abbreviations: GnRHR: GnRH receptor; ɾGFP: tau-green fluorescent protein; MOB: main olfactory bulb; AOB: accessory olfactory bulbs; PC: piriform cortex; OT: olfactory tubercle; EC: entorhinal cortex; BNST: bed nucleus of the stria terminalis; EPl, accessory olfactory bulb external plexiform layer; MI, mitral cell layer in the AOB; AOB GrL, accessory olfactory bulb granular cell layer; MOB GrL, main olfactory bulb granular cell layer; PC, piriform cortex; OT, olfactory tract; EC, entorhinal cortex; aMeA, Medial Amygdala anterior part; pdMeA, Medial Amygdala, posterior part.

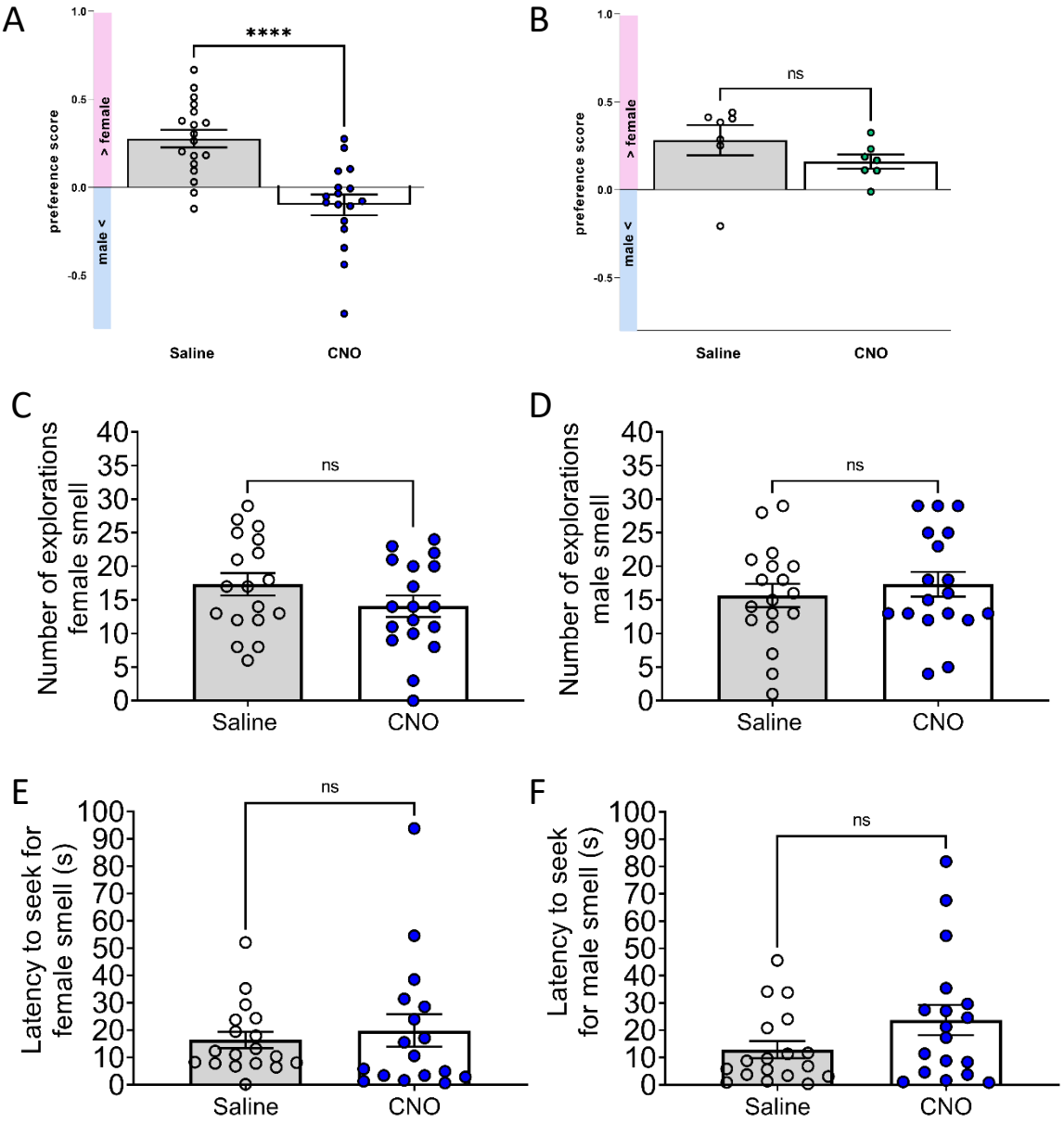

Figure 14: OB GnRH neurons projecting to the pdMeA are mediating olfactory preference. Olfactory preference test, performed as reported in figure 9B. CNO was injecting 10 min before the test. **(A)** Preference score of GnRH$^{cre/+}$ male mice infected with AAV9-hSyn-DIO-hM4D(Gi)-mCherry in the pdMeA. Paired t-test. **(B)** Preference score of GnRH$^{cre/+}$ male mice infected with AAV9-hSyn-DIO-mCherry in the pdMeA. Paired t-test. **(C-D)** Total number of (C) female and (D) male smell explorations during the 10 min olfactory preference test. Paired t-test. **(E-F)** Latency to seek for (E) female and (F) male smell in seconds. Wilcoxon test. N = 18 GnRH^{hM4Di}; N = 9 GnRH^{mcherry}; 12-16 weeks-old males; ns $P > 0.05$; **** $P < 0.0001$.

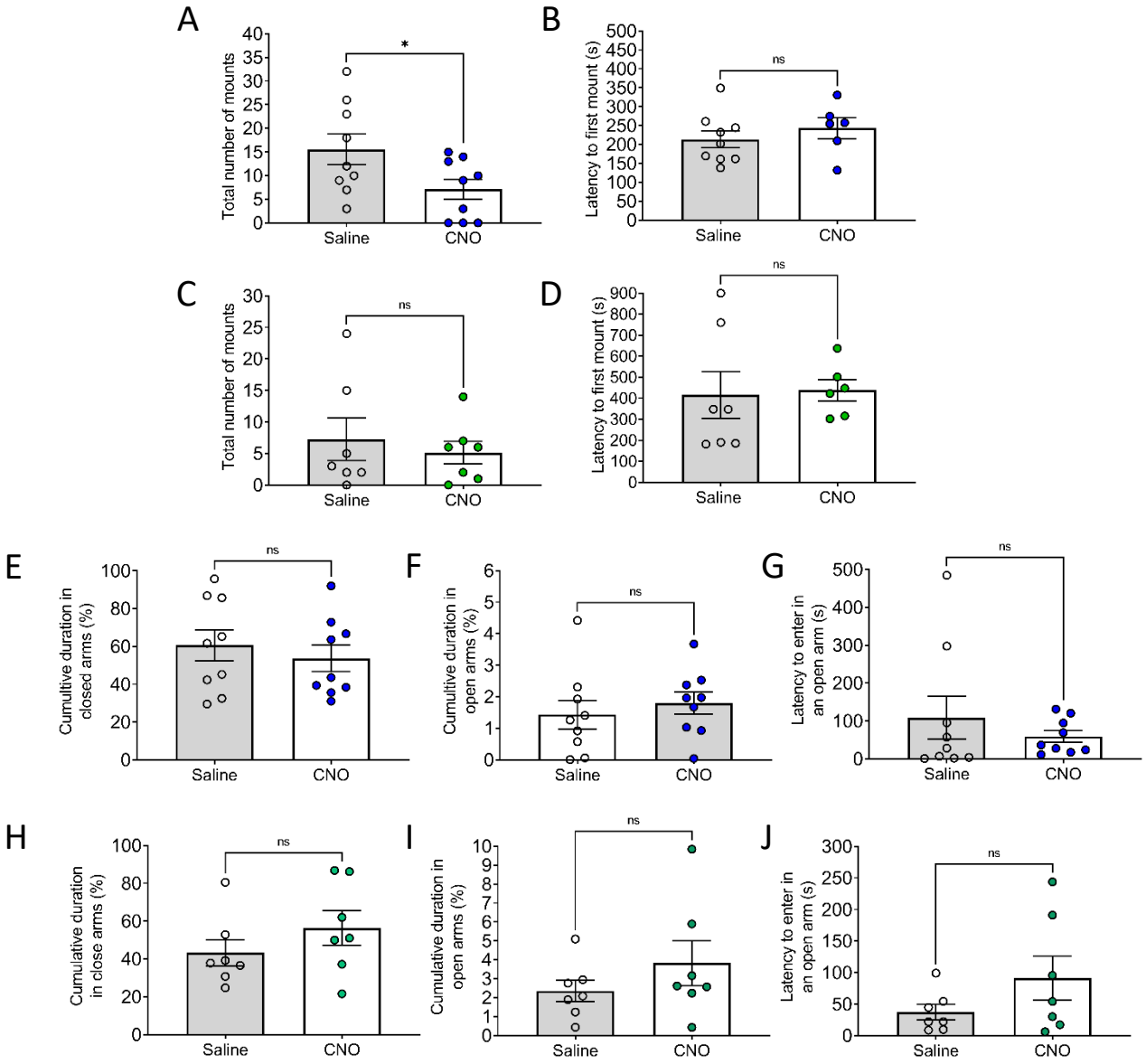

Figure 15: OB GnRH neurons projecting to the pdMeA modulate sexual behavior but not anxiety in male mice. Sexual behavior test and EPM test are performed in male mice that received either AAV9-hSyn-DIO-hM4D(Gi)-mCherry (GnRH^{hM4Di}) or AAV9-hSyn-DIO-mCherry (GnRH^{mcherry}) injections in the pdMeA. CNO and saline were injected 10 min before the test. **(A)** Total number of mounts and **(B)** latency to first mount during sexual behavior test in naïve in GnRH^{hM4Di} male mice after saline or CNO injections. **(C)** Total number of mounts and **(D)** latency to first mount during sexual behavior test after saline or CNO injections in naïve GnRH^{mcherry} male mice. **(E)** Cumulative duration in closed and **(F)** open arms and **(G)** latency to enter in open arm during the EPM test in GnRH^{hM4Di} male mice after saline or CNO injections. **(H)** Cumulative duration in closed and **(I)** open arms and **(J)** latency to enter in open arm during the EPM test in GnRH^{mcherry} male mice after saline or CNO injections. N = 9 GnRH^{hM4Di}; N = 9 GnRH^{mcherry}; 12-16 weeks-old males; Paired t-test; ns $P > 0.05$; * $P < 0.05$.

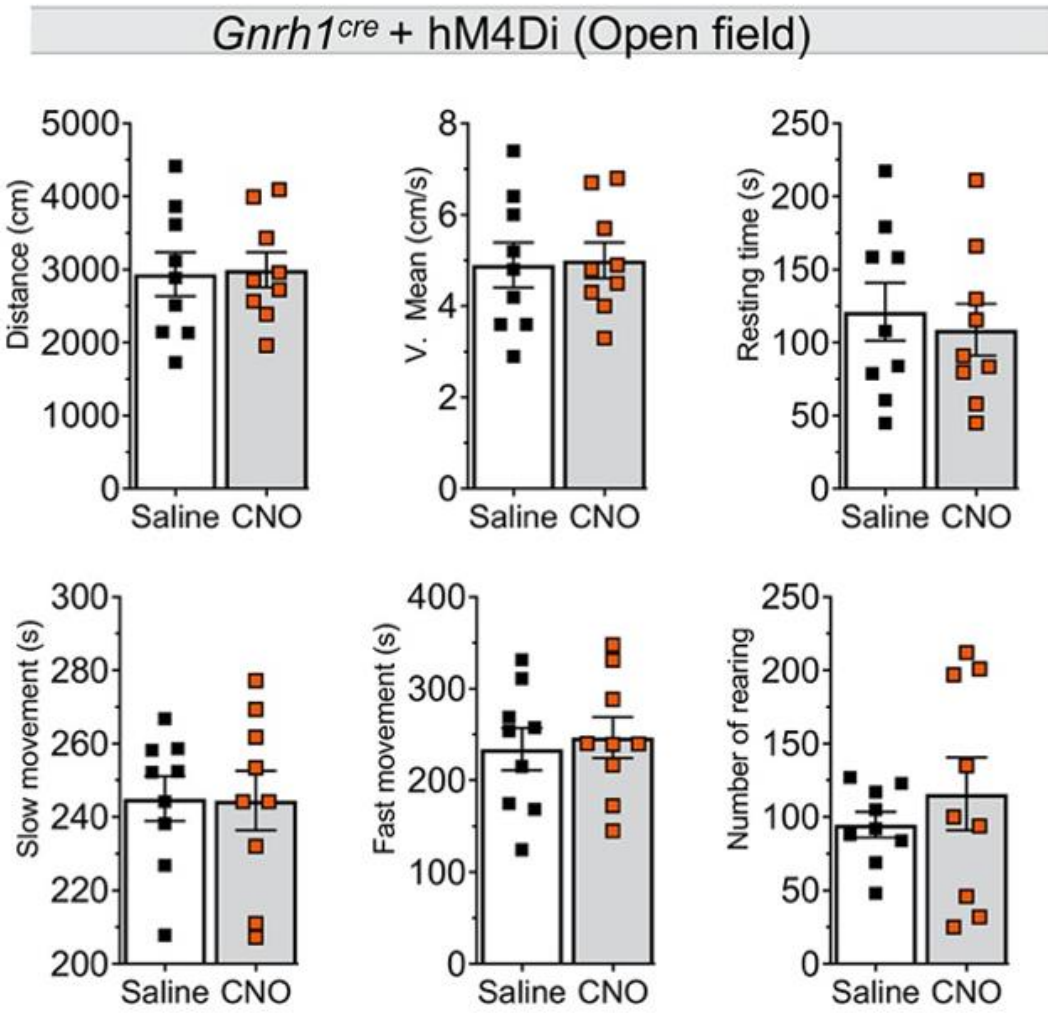

Figure 16: The inhibition of the OB GnRH neurons projecting to the medial amygdala does not modify the exploratory behavior in male mice. Graphs showing the different parameters evaluated during the open field test after chemogenetic inhibition of OB GnRH neurons projecting to the medial amygdala. N=9; 12-20 weeks-old males; ns $P > 0.05$; non-parametric Wilcoxon signed rank test. Values are represented as mean ± SEM.

A population of kisspeptin neurons have been identified in the MeA, which showed reciprocal connectivity with the AOB and integrate odor information to coordinate gonadotrophin release and behavioral output (Adekunbi et al., 2018; Aggarwal et al., 2019; Pineda et al., 2017). We thus performed RNAscope to evaluate whether kisspeptin neurons in the pdMeA may express the GnRH-R. Our data demonstrated that only MeA^{Kiss} neurons express the *Gnrhr* (**Figure 17A-B**) but not the ARC^{Kiss} or the $rPOA^{Kiss}$ neuronal populations (**Figure 17C-F**). We also assessed whether the *Gnrhr* expression may change as a function of sexual experience. Our data showed that sexual experience does not impinge on the expression of *Gnrhr* in any of the investigated areas and that in male mice nearly 70% of kisspeptin neurons located in the pdMeA express the *Gnrhr* (N=4 sexually naïve male mice, 66.67 ± 5.143; N=4 sexually experienced male mice, 71,43 ± 6,148; **Figure 17G**).

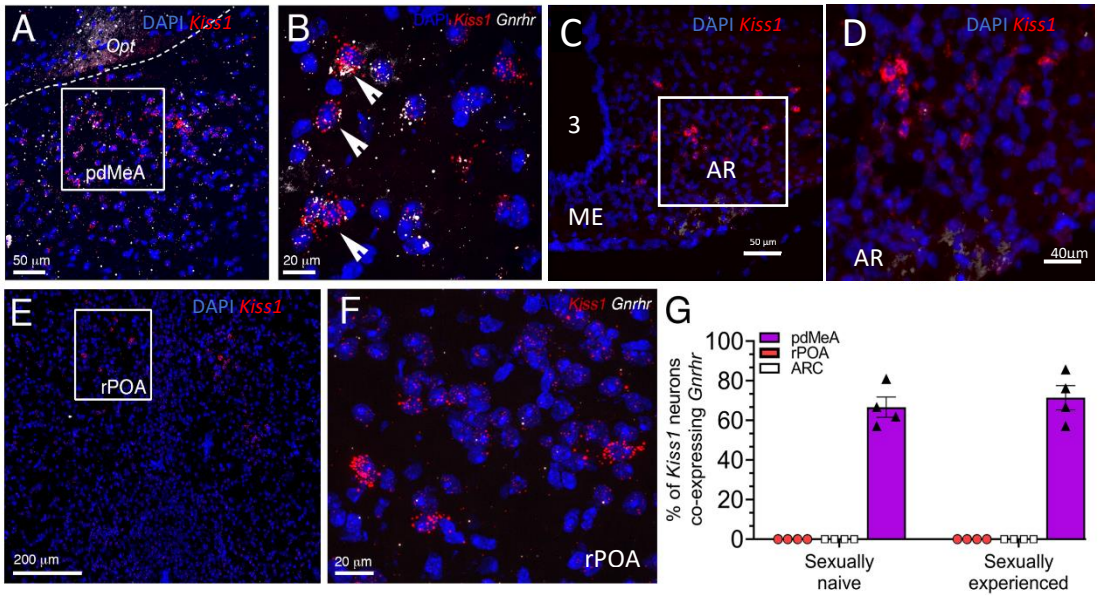

Figure 17: pdMeA kisspeptin neurons express GnRH-R. (A-B) Representative coronal section of the pdMeA from an adult male mouse (P90) showing expression of *Kiss1* mRNA (red staining) and *Gnrhr* (white staining) by RNAscope. The white dash line indicates the optic track (Opt). Arrows point to kisspeptin neurons co-expressing *Gnrhr*. **(C-D)** Representative coronal section of the ARC from an adult male mouse (P90) showing expression of *Kiss1* mRNA (red staining) and *Gnrhr* (white staining) by RNAscope. **(E-F)** Representative coronal section of the rPOA from an adult male mouse (P90) showing expression of *Kiss1* mRNA (red staining) and *Gnrhr* (white staining) by RNAscope. **(G)** Percentage of kisspeptin neurons co-expressing *Gnrhr* in the pdMeA, the rPOA, the ARC of sexually naïve and sexually experienced males. N = 4 naïve male mice and N = 4 sexually experienced male mice were used for FISH experiments.

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Chapter 6: Discussion and conclusion

Discussion

The aim of this study was to unveil the neuroanatomical features and functional role of OB GnRH neurons in the modulation of gonadotropin secretion and socio-sexual behaviors. To this aim, we combined viral approaches with tissue clearing, calcium imaging, electrophysiology, and behavioral tests. Our study demonstrates an unprecedented role for the olfactory GnRH system as a specialized hub uniting chemosensory cues with opposite-sex attraction, sexual behavior, and corresponding endocrine changes in male mice.

OB GnRH neurons detect olfactory cues

In this work, we showed that nearly 20% of the entire population of GnRH neurons is located in the olfactory bulbs with the majority of OB GnRH cell bodies located within the main olfactory bulb. In the main olfactory bulb, a high proportion of GnRH neurons is found in the olfactory nerve layer, where GnRH neurons are round-shaped and clustered. These neurons exhibit similar morphology to immature GnRH neurons during their migratory journey. The role of this sub-population of GnRH neurons as part of the olfactory bulb GnRH population is questionable since they do not extend processes and, as a result, are not integrated within the OB GnRH neuronal population. It is quite intriguing to find these neurons within the olfactory bulbs of adult male mice as the GnRH neuronal migration is known to terminate at birth. More investigations are currently ongoing in the lab to determine whether these neurons remain immatures and whether they are still migrating. Within the MOB, OB GnRH neurons are also located in the glomerular layer, an area known to integrate olfactory information and in the mitral and granular layers, areas involved in the relay of olfactory information. Moreover, GnRH neurons are also present within the different layers of the accessory olfactory bulb, within the anterior olfactory nucleus and the tenia tecta, two areas involved in olfactory information processing. The position of these olfactory GnRH neurons places them as a central hub potentially integrating and relaying olfactory information from both the main and the accessory olfactory systems.

A unique aspect of our study was the characterization of the OB GnRH population and direct visualization of the pattern of their projections toward the olfactory/vomeronasal structures and the hypothalamic regions regulating neuroendocrine responses. Indeed, whole-head immunostaining and OB GnRH viral tracing, provide evidence of anatomical connectivity between OB GnRH neurons and the olfactory and vomeronasal epithelia. OB GnRH neurons send direct projections to the vomeronasal organ. These olfactory GnRH neurons could relay olfactory information to the main and accessory olfactory systems and the hypothalamus. Research has demonstrated that a sharp increase in LH release was induced by intact estrous female urine in male mice (Maruniak and Bronson, 1976). OB GnRH neurons, located in the olfactory bulb, may perceive and relay this olfactory information to central brain areas, potentially playing a crucial role in the pheromone-induced LH surge exhibited by male mice upon encountering sexual partners. In addition, exposure to olfactory cues from the opposite-sex maintains downstream secretion of testosterone, highlighting the crucial role of OB GnRH neurons in the mediation of neuroendocrine responses following olfactory cues exposure.

Pheromones detection by the VNO mediates social behaviors, including sexual behavior. Our transcriptomic data point out the expression of 14 *Vmnrs* in 2 to 6% of OB GnRH neurons and 26 *Olfrs* in 2.5 to 12.5% of OB GnRH neurons, suggesting that a fraction of these cells may directly detect odorant and pheromonal molecules. Some of the vomeronasal receptors expressed by OB GnRH neurons have been previously identified as receptors involved in responses to female (*Vmn2r57*) and male (*Vmn2r84*, *Vmn2r85*, *Vmn2r86*, *Vmn2r87*) specific signals in males and involved in the detection of predator cues (*Vmn2r55*) (Isogai et al., 2011). The identification of female and male olfactory cues may play a role in socio-sexual behaviors. Additionally, OB GnRH neurons express both *Vmn2r1* and *Vmn2r2* mRNAs. Vomeronasal sensory neurons express receptors from both V1R and V2R families. The V2R family is divided into sub-families, with half of the basal vomeronasal sensory neurons co-expressing Vmn2r1 and the other half co-expressing Vmn2r2. The expression of Vmn2r1 and Vmn2r2 receptors by almost all vomeronasal sensory neurons suggests that pheromone responses are mediated through the interaction of one vomeronasal receptor and a specific receptor (Vmn2r1 and Vmn2r2), from the same family (Martini et al., 2001). The expression of both *Vmn2r1* and *Vmn2r2* mRNAs in OB GnRH neurons strengthens the hypothesis of pheromone detection by these neurons.

Finally, the RNAscope data indicate that olfactory and vomeronasal receptors are expressed concurrently by OB GnRH neurons in adult mice. Indeed, the mRNAs for both the vomeronasal receptor *Vmn2r1* and the olfactory receptor *Olfr323* are detected in the same OB GnRH neurons. The expression of more than one olfactory receptor is not detected in olfactory sensory neurons (Grabe and Sachse, 2018). Furthermore, vomeronasal receptors are exclusively co-expressed only in association with the Vmn2r1 and Vmn2r2 receptors (Martini et al., 2001). OB GnRH neurons may differ from the olfactory and vomeronasal sensory neurons. Additional analyses will be conducted to confirm these observations. Altogether, these data suggest that OB GnRH neurons can directly respond to olfactory stimuli.

Our hypothesis is supported by our *in vivo* two-photon functional imaging of OB GnRH neurons of males exposed to female estrous urine, which provided evidence that the activity of half of OB GnRH neuronal population can be modulated by exposure to opposite-sex urine. This evidence further strengthens the idea that OB GnRH neurons respond to olfactory cues and might transmit this olfactory information to downstream circuitry. Transcriptomic data showed that OB GnRH also express vomeronasal receptors responding to male specific signals in males, suggesting that OB GnRH neurons might be able to be activated by same-sex smells. Performing identical analysis using *in vivo* two-photon functional imaging and exposing male mice to male urine may address this question. However, it was previously reported that only opposite-sex smell triggered neuroendocrine responses (Maruniak and Bronson, 1976).

To date, this is the first *in vivo* evidence showing that mammalian GnRH neurons present odorant-driven activity, which was a missing piece in the puzzling role of these neurons in olfactory-mediated sex behaviors.

OB GnRH neurons mediate LH and testosterone release by activating POA GnRH neurons

We also found by virally-mediated circuit connectivity analysis that OB GnRH cells project to the hypothalamic preoptic area and to the median eminence. Interestingly, previous seminal studies have demonstrated that both the vomeronasal and main olfactory systems send direct or indirect projections to the hypothalamic GnRH neurons (Boehm *et al.*, 2005; Yoon *et al.*, 2005) and that chemosensory inputs (female urine) increase the activity of these cells located in the preotic area of adult male mice (Yoon *et al.*, 2005). Here, we found that OB GnRH neurons projecting to the hypothalamus (POA and ME) are mostly located in the main olfactory bulb and that chemogenetic activation of these cells results in a significant increase of the POA GnRH neuronal activity followed by LH and testosterone discharge. This response was completely abrogated after chemogenetic silencing of OB GnRH neurons. Therefore, consistently with previous studies, the LH release observed after opposite-sex smell exposure must be trigger by OB GnRH neurons (Maruniak and Bronson, 1976; Dluzen *et al.*, 1981).

Our original evidence also shows that this direct connections between olfactory sensing organs to neuroendocrine output is a way in which LH secretion might be rapidly triggered bypassing further limbic and cognitive processing by other hypothalamic nuclei and cortical areas. Because our transcriptomic data also showed that OB GnRH neurons express higher levels of GABAergic and glutamatergic markers as compared to hypothalamic POA GnRH neurons, such as *Grin1*, *Grin2b*, *Gad1* and *Gad2*, we speculate that OB GnRH cells may increase POA GnRH electrical activity through excitatory neurotransmission either directly and, or indirectly through actions on the POA GnRH afferent network. Unlike most adult neurons in the brain, hypothalamic GnRH neurons can respond with excitation to GABA via the activation of GABA type A receptors ($GABA_AR$)(DeFazio and Moenter, 2002). Moreover, the activation of specific hypothalamic GABAergic circuits has been shown to enhance GnRH/LH secretion (Silva et al., 2019). Altogether, these integrated approaches show that OB GnRH neurons decode the opposite sex chemosensory cues into activation of POA GnRH neurons, thus triggering fertility-related neuroendocrine changes.

OB GnRH neurons mediate olfactory preference

Chemosensory cues are known to stimulate male sexual arousal and behavior with the main olfactory system playing an important role in attracting males to estrous females, and the vomeronasal receptors activating accessory olfactory pathways that engage copulatory behavior due to its indirect connections with the reproductive hypothalamus (Keverne, 2004). Consistently, targeted deletion of the olfactory sensory transduction molecules downstream of odorant receptors, *CNGA2* (Mandiyan *et al.*, 2005) or *AC3* (Wang *et al.*, 2006) genes, which are expressed in the main olfactory epithelium but not in the vomeronasal organ, disrupts the preference for female urine odors in male mice and impairs male–female mating behavior. A previous work by Hellier and colleagues showed that *Gnrh1*$^{cre/wt}$*;Dicer*$^{loxP/loxP}$ females, which are incapable of synthesizing and secreting GnRH in adulthood (Messina *et al.*, 2016), failed to show male-directed preferences (Hellier et al., 2018). Data from the lab expanded and corroborated these findings by demonstrating that GnRH-deficient males also fail to show female-directed preferences. Strikingly, a single GnRH injection reversed the olfactory discrimination impairment toward opposite sex-smell in both *Gnrh1*$^{cre/wt}$*;Dicer*$^{loxP/loxP}$ males and females (unpublished data). These findings were further corroborated by our experiments showing that chemogenetic inhibition or ablation of OB GnRH neurons disrupts the social olfactory preference of adult males, highlighting the role of this GnRH population in the drive of olfactory preference. However, while chemogenetic silencing of OB GnRH neurons completely blunted the gonadotropic response and LH secretion, the taCasp3-mediated ablation of about 40% of the OB GnRH neuronal population did not impact LH secretion whereas it was sufficient to alter the olfactory preference of adult *Gnrh1*$^{cre/wt}$ male infected mice. These data suggest that the control of gonadotropin release is very robust, and likely controlled by redundant circuits, and it requires the contribution of the majority of the OB GnRH neuronal population, while subtle changes in the number of these neurons can lead to significant behavioral changes in social recognition.

OB GnRH neurons modulate motivation to seek for opposite-sex odorants and facilitate the initiation of sexual behavior in naïve males

Consistent with the expression of GnRH and its receptor in several extrahypothalamic areas in mice and humans (Casoni *et al.*, 2016; Manfredi-Lozano et al., 2022; Schang et al., 2011; Skrapits et al., 2021; Wen et al., 2011), our mapping experiments revealed a wide distribution of GnRH-R expressing cells in the main and accessory olfactory systems as well as along all cortical and hypothalamic recipients of the MOB and AOB. Interestingly, the preference exhibited by adult mice toward the opposite sex-smell is known to be mediated by a cooperation between the main and accessory olfactory systems (Hurst, 2009) with the MOE making a greater contribution to the preference for the opposite sex and initiation of sexual behavior, and the VNO signaling involved in gender identification (Choi *et al.*, 2016; Keverne, 2004; Mandiyan *et al.*, 2005). Interestingly, we showed that the ablation of 40% of the OB GnRH neuronal population reduces the ability to detect social olfactory cues and attenuates the motivation to seek for opposite-sex odorants in male mice. However, mate preference did not change in the presence of conspecifics.

Additionally, a recent study highlighted the importance of the GnRH-R in sexual motivation and reward in male rats. The injection of Degarelix, the GnRH-R antagonist, attenuates copulatory ability and sexual performance (Hawley et al., 2021). In this study, we found that the 40% reduction of the OB GnRH population only impairs copulatory behavior in naïve males. Due to the interconnections between the olfactory and the reproductive systems, the presence of the GnRH system may be an essential component driving the initiation of sexual behavior. Besides, focusing on the GnRH-R expressing cells, we demonstrated that female urine stimulation enhances the activation of GnRH-R expressing cells in the olfactory bulbs, the postero-dorsal part of the medial amygdala and the piriform cortex, which are important brain regions downstream of the main and accessory olfactory systems. For instance, the medial amygdala has been shown to be involved in the copulatory behavior in naïve males and could be a downstream target of OB GnRH neurons driving sexual behavior.

To conclude, the presence of the GnRH system along olfactory areas improves the detection of social olfactory cues in order to facilitate the initiation of the copulatory behavior in male mice. Mating behavior is then triggered by central brain areas receiving olfactory inputs.

Downstream targets of OB GnRH neurons

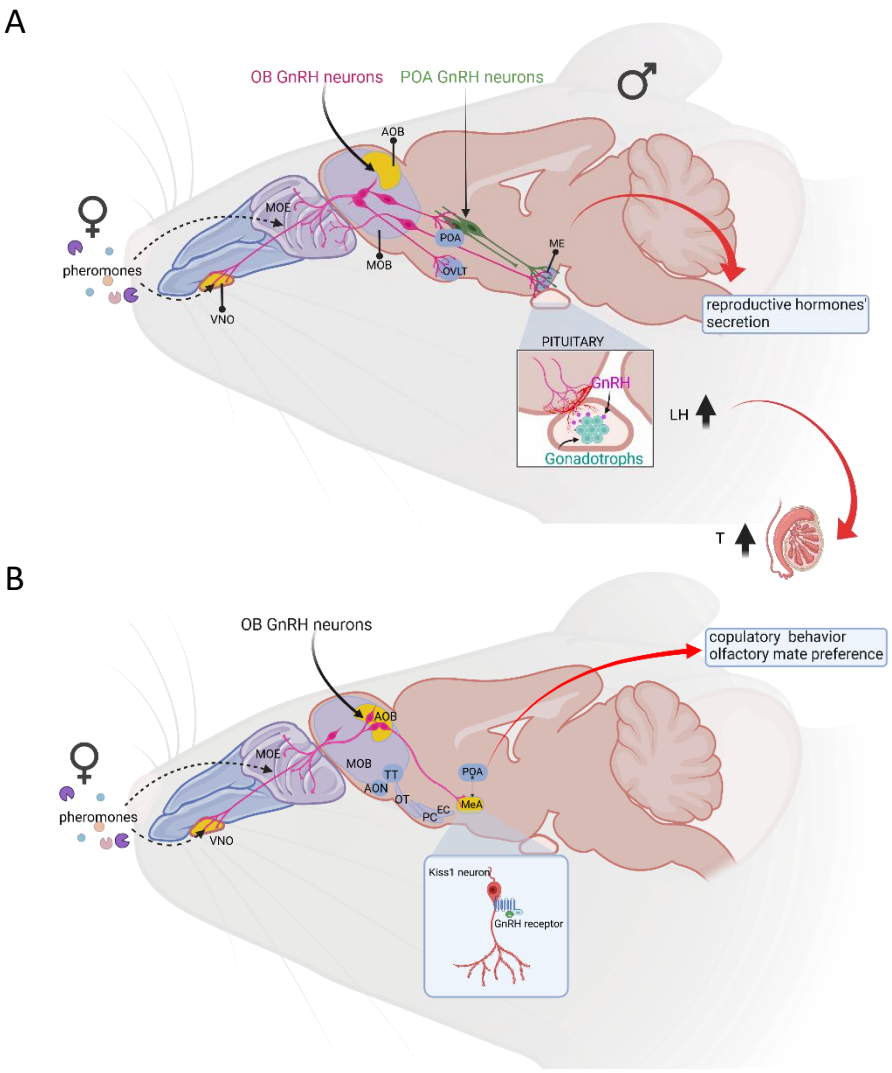
The neurons of the pdMeA are integrated in a neural network involved in sexual behavior, as this limbic center participates in interpreting olfactory chemosignals and mediates partner preference (Kondo, 1992). A certain degree of convergence exists between the main and accessory olfactory systems at the level of the cortico-medial amygdala (Gomez and Newman, 1992; Kevetter and Winans, 1981a; b; Meredith, 1998), thus suggesting that pheromonal signals mediated through both systems could interact and impact the downstream network involved in the regulation of sexual behavior. Several articles demonstrate the enhancement of main olfactory input to the medial amygdala through the involvement of GnRH and an extra-hypothalamic kisspeptin neuronal population (Blake and Meredith, 2010; Pineda et al., 2017; Adekunbi et al., 2018; Lehman and Winans, 1982; Wood and Newman, 1995). However, the identity of cells relaying signals from the olfactory system to the medial amygdala to finally modulate sex recognition and mating behavior remains poorly defined. In this study, we provide evidence that both OB GnRH terminals and GnRH-R expressing cells are present in the pdMeA and that these GnRH-R expressing cells are activated after opposite sex-smell exposure. In addition, chemogenetic inhibition of OB GnRH neurons projecting specifically to the medial amygdala significantly abrogated the olfactory preference of sexually naïve *Gnrh1*$^{cre/wt}$ male mice toward the female urine and impacted their copulatory behavior, thus suggesting that OB GnRH neurons are essential for both sex recognition and the initiation of mating behavior in naïve males. We did not observe altered anxiety behavior or locomotor activity in GnRH^{hM4Di} male mice in which we targeted the expression of the inhibitory DREADD virus into the pdMeA, implying that the OB GnRH neurons sending projection to the pdMeA are unlikely to exert a major influence on neural pathways associated with anxiety, on the contrary to the medial amygdala kisspeptin population, which is involved in both partner preference and anxiety in male mice (Adekunbi et al., 2018).

Kisspeptin neurons are essential for sexual behavior, indeed, kisspeptin receptor knockout mice display no sexual behavior. Recently, the kisspeptin population located within the medial amygdala has been demonstrated to enhance olfactory inputs to the medial amygdala through the reciprocal connections between the medial amygdala kisspeptin population and the olfactory bulbs and to mediate LH release (Blake and Meredith, 2010; Pineda et al., 2017; Adekunbi et al., 2018; Lehman and Winans, 1982; Wood and Newman, 1995). This kisspeptin population located in the medial amygdala has also been demonstrated to contact the POA GnRH neurons (Pineda et al., 2017) and the kisspeptin population located in the hypothalamus (Aggarwal et al., 2019), highlighting the presence of a circuit linking the accessory olfactory system with the preoptic area. Here, we demonstrated that a vast majority of kisspeptin neurons located in the pdMeA express the GnRH-R. Thus, this circuit may facilitate the initiation of reproductive behavior in naïve male mice as the MPOA seems to be the final node of sexual performance in males (Bayless et al., 2023). Gresham et al. (2016), corroborate the role of the kisspeptin population in male mice sexual behavior by demonstrating that kisspeptin administration in the postero-dorsal medial amygdala stimulates both erectile response and LH secretion.

Conclusion

This study provides novel insights into the biology and homeostasis of reproduction in mammals. The mechanism we observed in mice likely operates in other species as well, and a strong human relevance is suggested by the presence of GnRH neurons in the OB of human post-mortem brains shown in chapter 3. Quite convincing pheromonal effects have been demonstrated in humans who do not have a functional vomeronasal system (Meredith, 2001; Wysocki and Preti, 2004). Indeed, human axillary cues affect the timing of ovulation in women (Stern and McClintock, 1998) and LH pulsatile release (Preti et al., 2003). Additionally, these kinds of putative pheromonal signals induce brain activation only in humans with intact main olfactory function, as revealed by functional brain imaging (Savic et al., 2009). However, the issue of whether these responses are mediated by a functional VNO in humans is still controversial, although previous studies have demonstrated the existence of a distinguishable VNO in a substantial proportion of adult humans (Frasnelli et al., 2011) as well as in human embryos and fetuses (Casoni *et al.*, 2016).

During the last few years, we and others have started to shed light on to novel extra-reproductive roles of GnRH neurons, associated with cognitive functions (Manfredi-Lozano *et al.*, 2022) and programming of systemic aging (Zhang et al., 2013). The current study corroborates and expands those findings, highlighting a role for OB GnRH neurons in the processing of chemo-social cues responsible for opposite-sex attraction. Overall, our work raises the intriguing concept that GnRH neurons in the olfactory bulb act as a bridge between the different olfactory epithelia, the neuroendocrine hypothalamus, and the medial amygdala to synchronize the secretion of reproductive hormones and the behaviors induced by olfactory cues in males.



OB GnRH neurons drive endocrine and behavioral changes. (A) Schematic representation of MOB-GnRH neuronal projections to the MOE, the VNO, the OVLT, the POA GnRH neurons and the ME. MOB-GnRH neurons are activated after exposure to olfactory cues and in response stimulate gonadotropic axis by enhances LH and testosterone secretions. MOB-GnRH neuronal activation also increases the firing rate of the rPOA GnRH neurons. These two populations may participate together to control reproductive hormones' secretion after opposite-sex smell exposure. **(B)** Schematic representation of AOB-GnRH neuronal projections to the MOE, the VNO and to the MeA. In response to opposite-sex smell exposure, AOB-GnRH neurons drive olfactory mate preference and facilitate the initiation of copulatory behavior in naïve male mice through the GnRH-R express by the pdMeA kisspeptin neurons. *VNO: vomeronasal organ; MOE: main olfactory epithelium; MOB: main olfactory bulbs; AOB: accessory olfactory bulbs; POA: preoptic area; OVLT: organum vasculosum of the lamina terminalis; ME: median eminence; LH: luteinizing hormone; T: testosterone; TT: tenia tecta; AON: accessory olfactory tubercle; OT: olfactory tubercle; PC: piriform cortex; EC: entorhinal cortex; MeA medial amygdala.*

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Annexes

Refereed Journal Articles

PNAS RESEARCH ARTICLE | NEUROSCIENCE OPEN ACCESS

Female sexual behavior is disrupted in a preclinical mouse model of PCOS via an attenuated hypothalamic nitric oxide pathway

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Women with polycystic ovary syndrome (PCOS) frequently experience decreased sexual arousal, desire, and sexual satisfaction. While the hypothalamus is known to regulate sexual behavior, the specific neuronal pathways affected in patients with PCOS are not known. To dissect the underlying neural circuitry, we capitalized on a robust preclinical animal model that reliably recapitulates all cardinal PCOS features. We discovered that female mice prenatally treated with anti-Müllerian hormone (PAMH) display impaired sexual behavior and sexual partner preference over the reproductive age. Blunted female sexual behavior was associated with increased sexual rejection and independent of sex steroid hormone status. Structurally, sexual dysfunction was associated with a substantial loss of neuronal nitric oxide synthase (nNOS)-expressing neurons in the ventromedial nucleus of the hypothalamus (VMH) and other areas of hypothalamic nuclei involved in social behaviors. Using in vivo chemogenetic manipulation, we show that $nNOS^{VMH}$ neurons are required for the display of normal sexual behavior in female mice and that pharmacological replenishment of nitric oxide restores normal sexual performance in PAMH mice. Our data provide a framework to investigate facets of hypothalamic nNOS neuron biology with implications for sexual disturbances in PCOS.

PCOS | AMH | sexual behavior | nitric oxide | hypothalamus

Significance

Polycystic ovary syndrome (PCOS) is a major endocrine disorder leading to female infertility worldwide. Patients with PCOS also often experience sexual dysfunction; however, the developmental and central mechanisms mediating this behavioral derangement are unclear. Here, we show that prenatal excess of anti-Müllerian hormone triggers PCOS-like impairment in female sexual behavior in mice. Sexual dysfunction in PCOS-like mice is associated with decreased expression of progesterone-sensitive neuronal nitric oxide synthase (nNOS) neurons in the hypothalamus. Chemogenetic inhibition of nNOS neuronal activity in the ventromedial nucleus of the hypothalamus recapitulates PCOS-like sexual dysfunction. Of clinical relevance, administration of nitric oxide donor rescues normal sexual behavior in PCOS-like mice.

Polycystic ovary syndrome (PCOS) is a highly prevalent disease affecting 5 to 18% of women of reproductive age worldwide (1, 2). PCOS is diagnosed upon the presence of at least two out of three prime features: high circulating levels of androgens (hyperandrogenism), menstrual irregularities (oligo-anovulation), and polycystic-like ovarian morphology (2, 3). Beyond its implications leading to female infertility, the disease is associated with several metabolic disruptions, cardiovascular diseases, and psychosocial disorders (4). Among these neurological implications, it has become clear that approximately 30% or more of patients with PCOS experience sexual dysfunctions, with clinical studies reporting a high risk of low sexual arousal, desire, and satisfaction and impaired lubrication and orgasm (5–9). These symptoms allude to disturbances in brain circuits controlling sexual function in the context of PCOS.

Neural circuits driving female sexual behaviors are conserved among vertebrate species operating under the influence of sex steroid hormone modulation, which is paramount for partner interaction, receptivity, and sexual performance (10, 11). Indeed, gonadal sex hormones are implicated in shaping circuit architecture in the hypothalamus during development and activating these neonatally programmed circuits over reproductive adult life in many species (12–16). The hypothalamus integrates sensorial stimuli and autonomic arousal from endogenous sex drive cues (e.g., estrous phase, energy status, hormone milieu, genital stimulation) to convey this information to other brain areas and peripheral nerves (10, 17). The ventromedial nucleus of the hypothalamus (VMH) is considered the hub of specialized neurons, with intrinsic properties driving different components of sexual behavior (18–21). The VMH harbors neurons expressing neuronal nitric oxide synthase (nNOS), the enzyme responsible for the production of nitric oxide (NO), a key gaseous neurotransmitter that stimulates female sexual behavior (22, 23) and communicates with other circuits within the social brain (24, 25). Despite current advances unraveling novel pathways in the female sexual brain with specific behavioral responses, there is a clear lack of knowledge on how disturbances in these circuits may participate in sexual dysfunctions affecting one-third of women with PCOS.

Growing evidence indicates that androgen excess *in utero* induces a developmental reprogramming of the female fetal brain toward the manifestation of PCOS traits later in life (26–29). Some studies have suggested that the clinical signs of hyperandrogenism

Competing interest statement: P.G., M.S.B.S, K.C., and V.P. disclose that they are inventors of a submitted patent application by the Institut National de la Santé et de la Recherche Médicale (INSERM) covering methods for treatment of sexual dysfunctions in PCOS.

This article is a PNAS Direct Submission.

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RESEARCH ARTICLE SUMMARY

NEURODEVELOPMENT

GnRH replacement rescues cognition in Down syndrome

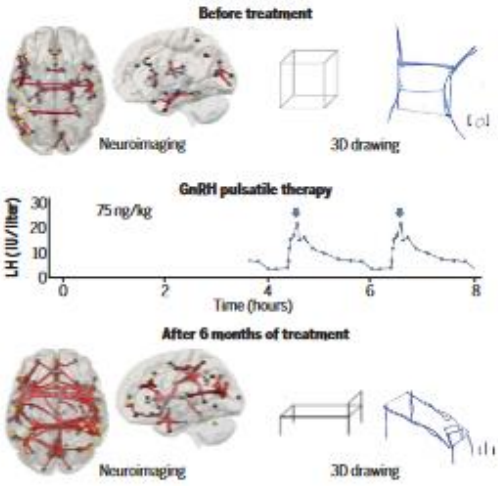
Maria Manfredi-Lozano†, Valerie Leysen†, Michela Adamo†, Isabel Paiva, Renaud Rovera, Joan-Michel Pignat, Fatima Ezzahra Timzoura, Michael Candlish, Sabiha Eddarkaoui, Samuel A. Malone, Mauro S. B. Silva, Sara Trova, Monica Imbernon, Laurine Decoster, Ludovica Cotellessa, Manuel Tena-Sempere, Marc Claret, Ariane Paoloni-Giacobino, Damien Plassard, Emmanuelle Paccou, Nathalie Vionnet, James Acierno, Aleksandra Maleska Maceski, Antoine Lutti, Frank Pfrieger, S. Rasika, Federico Santoni, Ulrich Boehm, Philippe Ciofi, Luc Buée, Nasser Haddjeri, Anne-Laurence Boutillier, Jens Kuhle, Andrea Messina, Bogdan Draganski, Paolo Giacobini†, Nelly Pitteloud*†, Vincent Prevot*†

INTRODUCTION: Patients with Down syndrome (DS), a frequent condition (1 per 800 live births) caused by trisomy of chromosome 21, display a variety of characteristics, including cognitive decline due to an early-onset Alzheimer-like disease along with myelination defects in adulthood, impaired olfaction starting before puberty, and subfertility. No viable treatment exists for the cognitive and olfactory deficits seen in DS patients.

RATIONALE: Gonadotropin-releasing hormone (GnRH), which is secreted in a pulsatile fashion by specialized hypothalamic neurons, is the master molecule that controls reproduction in all mammals. In humans, altered GnRH secretion leads to Kallmann syndrome, which manifests with olfactory defects, gonadal immaturity, and infertility. Hypothalamic GnRH-expressing neurons also project to extrahypothalamic areas, including those involved in intellectual functions. We therefore asked whether the progressive deficits observed in DS bore any temporal correlation to the maturation of the GnRH system; what alterations to this process, if any, could be observed in the brain of animal models of DS; and whether these alterations could be therapeutically reversed in adulthood.

RESULTS: We first further characterized a trisomic mouse model of DS that triplicates regions analogous to human chromosome 21 (Ts65Dn mice) and displays subfertility and progressive cognitive and olfactory impairments similar to that of DS patients. These nonreproductive neurological symptoms closely paralleled a postpubertal loss of GnRH neurons and fibers in the hypothalamus as well as in extrahypothalamic regions, which is reflected by changes in the levels and pattern of release of the gonadotropin luteinizing hormone (LH) in the blood. The decreased GnRH expression in adult mice was accompanied by an imbalance in a complex network of microRNAs (several of which occur on the trisomic region) and regulatory factors that constitute a "switch" that controls GnRH expression and GnRH neuron maturation in the hypothalamus, starting during the infantile period or "minipuberty." Indeed, we observed that elements of this switch were dysregulated as far back as the minipubertal period, well before the appearance of cognitive or olfactory deficits. Additionally, this altered expression of microRNAs and transcription factors in the hypothalamus appeared to result in the altered expression of a number of target genes, including several involved in myelination and synaptic transmission, both in the hypothalamus itself and, to a much greater extent, in the hippocampus, as well as in the altered activity of hippocampal neurons. Overexpressing a key microRNA involved in the GnRH developmental switch, miR-200b, in the hypothalamus abolished both the changes in gene expression and the deficits in neuronal activity, olfaction, and cognition in DS mice. Reinduction of miR-200b in adulthood, long after the GnRH switch, increased both the number of GnRH-expressing neurons in the hypothalamus and the proportion of neurons expressing one of its transcriptional activators, Otx2, which is known to control the opening and closing of other critical periods in brain maturation. To verify that these improvements were indeed due to the restoration of GnRH, we used cell therapy with normal hypothalamic neurons as well as chemogenetic and pharmacological interventions to produce GnRH at physiological levels and patterns (i.e., pulsatile secretion) in adult DS mice and found that these treatments all abolished olfactory and cognitive defects in the mice. Finally, based on these results, we performed a pilot study in DS patients to assess the effects of pulsatile GnRH therapy on olfaction, cognition, and brain structure and function. This treatment is safe and is presently used to treat GnRH-deficient conditions like Kallmann syndrome. We found that a 6-month pulsatile GnRH treatment improved both cognitive performance and functional brain connectivity in these patients.

CONCLUSION: The maintenance of the GnRH system appears to play a developmental role in brain maturation and higher functions. Pulsatile GnRH therapy holds promise to improve cognitive deficits in DS, paving the way for future clinical trials. ■



Pulsatile GnRH therapy improves cognition in DS. DS patients show olfactory and cognitive impairments in addition to intellectual disability and reproductive maturation deficits. GnRH neurons, which control reproduction, also project to brain areas involved in cognition such as the hippocampus. In trisomic Ts65Dn mice, which mimic characteristics of DS patients, GnRH expression progressively disappears. Pulsatile GnRH therapy in DS patients improves brain connectivity and function. IU/liter, international units per liter.

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